Diversity and Evolutionary Origin of the Virus Family *Bunyaviridae*

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Table of Contents

1. SUMMARY

The family *Bunyaviridae* is one of the largest groups of viruses and contains more than 350 taxa. Five genera are assigned to the family, namely *Hantavirus*, *Nairovirus*, *Orthobunyavirus*, *Phlebovirus*, and *Tospovirus*. Most bunyaviruses are transmitted by arthropods and share the common feature of dual host tropism for arthropods and vertebrates, with the exception of hantaviruses that are only found in mammals. Due to their role as agents of disease, bunyaviruses serve as suitable models to study vector-borne viruses. Until recently, viruses grouping outside the five known genera of the family *Bunyaviridae* were unknown. All bunyaviruses isolated from blood-feeding arthropods appeared to infect vertebrates.

This thesis describes the discovery and characterization of six novel viruses that were isolated from tropical mosquitoes collected in Africa. The viruses share all typical bunyavirus characteristics such as a tripartite negative-sense genome and a cap-snatching activity during viral transcription. Sequence identity to other members of the family *Bunyaviridae* was up to 25% in the highly conserved region of the RNA-dependent RNA polymerase (RdRp) gene. RdRp sequence distances of the six novel viruses were almost equidistant to each other and to all established genera. In phylogeny the viruses established four unique deep branching lineages that shared ancient common ancestors with viruses from the vertebrate-infecting bunyavirus genera. The new lineages were proposed to define four new genera within the family *Bunyaviridae*, tentatively named *Fera-*, *Gouko-*, *Herbe-*, and *Jonvirus*.

Gouko- and herbeviruses do not seem to encode the nonstructural proteins NSm and NSs which are present in their closest relatives, vertebrate infecting viruses of the genera *Phlebo-* and *Orthobunyavirus*, respectively. The NSs protein is an important pathogenicity factor and suppresses the antiviral immune response in vertebrates. In contrast to gouko- and herbeviruses, jon- and feraviruses encode an NSs protein which, however, uses a coding strategy that has not been described in bunyaviruses before. It should be further investigated whether the NSs protein is able to interfere with the host´s antiviral RNAi response, as it has been reported for tospoviruses. Host restrictions were tested by infecting cell cultures from birds, mammals and reptiles. None of the cell cultures was permissive for any of the six novel viruses, suggesting host range restriction to insects. Concordantly, all novel viruses replicated in cell culture at ambient temperature but not at vertebrate-typical temperatures. These data suggest that fera-, gouko-, herbe-, and jonviruses represent the first insect-specific members of the family *Bunyaviridae*. The evolution of host tropism in the family was analysed by ancestral state reconstruction of host associations at the bunyavirus root and at all major lineage bifurcations. The results support the hypothesis that the vertebrate-pathogenic arboviruses evolved from ancestors that exclusively infected arthropods. Based on paraphyletic distribution of dual host tropism, the ability to infect vertebrates seem to have evolved several times convergently during bunyavirus evolution.

Knowledge on the diversity and evolutionary origin of viruses may help to understand mechanisms of viral emergence and identify genes necessary for the infection of novel host species.

2. LIST OF PUBLICATIONS

The data presented in this thesis were published in field specific and interdisciplinary peer-reviewed journals:

1. **Marklewitz, M.**, Handrick S., Grasse W., Kurth A., Lukashev A., Drosten C., Ellerbrok H., Leendertz F.H., Pauli G., Junglen S.; Gouléako virus isolated from West African mosquitoes constitutes a proposed novel genus in the family *Bunyaviridae*. **Journal of Virology**, 2011 Sep, 85(17):9227-34 - M. Marklewitz & S. Handrick equal contribution

   Key findings:
   The family Bunyaviridae comprises five genera of viruses infecting humans, animals and plants. In this publication a virus of a proposed novel bunyavirus genus is characterized. The virus named Gouléako virus (GOLV) was isolated from mosquitoes and shows all bunyavirus characteristics. However, its genome is shorter than that of all previously known bunyaviruses and lacks accessory genes. GOLV is phylogenetically placed in basal relationship to the vertebrate infecting phleboviruses. GOLV is not able to infect cell lines derived from a wide range of vertebrate hosts suggesting that its host range is restricted to insects.

2. **Marklewitz M.**, Zirkel F., Rwego I.B., Heidemann H., Trippner P., Kurth A., Kallies R., Briese T., Lipkin W.I., Drosten C., Gillespie T.R., Junglen S.; Discovery of a unique novel clade of mosquito-associated bunyaviruses. **Journal of Virology**. 2013 Dec, 87(23):12850-65 - M. Marklewitz & F. Zirkel equal contribution

   Key findings:
   In this study another proposed new genus in the family Bunyaviridae is described. The viruses namely Herbert- (HEBV), Kibale- (KIBV) and Taï virus (TAIV) were isolated from mosquitoes of tropical Africa and represent a highly diversified group of viruses that define a monophyletic sister clade to the genus Orthobunyavirus. HEBV, KIBV and TAIV are phylogenetically equidistant to the human and animal pathogenic viruses of the genus Orthobunyavirus and to the plant infecting viruses of the genus Tospovirus. None of the viruses was able to infect vertebrate cells suggesting that these viruses represent the second group of insect-specific viruses within the family Bunyaviridae.

3. Junglen S., **Marklewitz M.**, Zirkel F., Wollny R., Meyer B., Heidemann H., Metzger S., Annan A., Dei D., Leendertz F.H., Oppong S., Drosten C.; No Evidence of Gouléako and Herbert Virus Infections in Pigs, Côte d'Ivoire and Ghana. **Emerging Infectious Diseases**. 2015 Dec, 21(12):2190-3

Key findings:

In 2014, South Korean scientists claimed that the prototype viruses of two recently described new insect-specific bunyavirus genera, HEBV and GOLV, were responsible for lethal infections in Korean pigs (Chung et al., 2014). Due to the experimentally verified insect-specificity of GOLV and HEBV, this publication attempted verification. Serum samples, collected from pigs at the same locality where GOLV and HEBV were initially discovered, were tested for present and past infections with GOLV and HEBV. No evidence for infections of pigs with GOLV and HEBV was found. Vertebrate cell culture infection experiments performed by Chung and coworkers were repeated with prototype strains of GOLV and HEBV. In contrast to the findings of Chung et al, no viral replication in vertebrate cell was detected. The Koeran strains fall within the genetic diversity of insect-specific GOLV and HEBV strains and are not likely to represent phylogenetic outliers that might have acquired a vertebrate tropism. As a consequence, GOLV and HEBV are highly unlikely to be responsible for the lethal disease outbreak in South Korean pigs.

4. **Marklewitz M.**, Zirkel F., Kurth A., Drosten C., Junglen S.; Evolutionary and phenotypic analysis of live virus isolates suggests arthropod origin of a pathogenic RNA virus family. **Proceedings of the National Academy of Sciences of the United States of America**. 2015 Jun, 112(24):7536-41 - M. Marklewitz & F. Zirkel equal contribution

   Key findings:

   The evolutionary origin of arboviruses remains unknown. Knowledge on their evolution can provide important insights into the emergence of pathogenicity. In this study two distinct novel insect-specific lineages of the human-, animal-, and plant pathogenic family Bunyaviridae are characterised. Both lineages branch in basal phylogenetic relationship to hantaviruses, the only genus that is not transmitted by arthropod vectors. The viruses termed Ferak- and Jonchet virus are incapable of replicating in vertebrate cells but grow to high titers in insect cells. This expansion of knowledge on the diversity of insect-specific bunyaviruses, combined with ancestral state reconstruction of ancient host associations, revealed arthropod-specific viruses as ancient progenitors of all major vertebrate infecting bunyavirus lineages.

3. GENERAL INTRODUCTION

3.1 Insect Viruses

The class *Insecta* represents one of the oldest and the most successful groups in the evolution of life (Misof *et al.*, 2014). Insects are classified into nearly one million distinct species and represent half of all living organisms on earth. Except for the seas, they colonise all ecological niches including polar regions. Their origin has been dated back to the early Ordovician (approximately 479 million years ago) and the emergence of insect flight to the early Devonian (approximately 406 million years ago) (Misof et al. 2015).

Due to their long existence insects may have coped with an extremely large variety of pathogens over millions of years. Consequently insects have developed mechanisms to resist these infections. The insect immune system is considered as an evolutionary root of the mammalian innate immune system (Vilmos & Kurucz, 1998). A central pathway to encounter viral infections is based on a nucleic acid based, post-transcriptional gene regulation process called RNA interference (RNAi). However, insect-infecting viruses developed proteins that inhibit the RNAi pathway [refer to 3.3.3].

As proposed by Junglen & Drosten (2013) the true genetic diversity of insect-associated viruses is still unknown as until now the focus was on those viruses that infect blood-feeding insects and can be transmitted to vertebrates. Recently arthropod transcriptome sequencing gave insight into the diversity of insect-associated viruses and their evolution [refer to 3.4]. The analyses of arthropod transcriptomes reveal a previously unknown diversity of viruses (Li *et al.*, 2015). The phylogenetic analysis of discovered insect-associated viruses showed a basal relationship to virus genera holding viruses that are highly relevant for human, animal and plant health. None of the insect-specific viruses falls into the diversity of known vertebrate or plant infecting viruses (Li *et al.*, 2015). These findings can be considered as the 'tip of the iceberg' in discovering the diversity of insect-associated viruses.

3.2 Arboviruses

The term arbovirus is an acronym which is derived from arthropod-borne virus and it combines a non-taxonomic group of viruses sharing the unique feature of the ability two arthropods and vertebrates as two disparate hosts (dual host tropism). More than 350 arboviruses are known where most of them are zoonotic or have a zoonotic potential (Centers for Disease Control and Prevention, 2016). The distribution of arboviruses is showing a hotspot in tropical regions but these viruses are also present in temperate regions. Arboviruses can replicate in a homeotherm vertebrate with a complex innate and adaptive immune system and in a poikilotherm arthropod with an RNAi-based (among others) immune system lacking a long-term memory effect. The transmission of arboviruses between arthropod vectors and vertebrate hosts occurs during the arthropods blood-feeding process. The range of susceptible vertebrate hosts and arthropod vectors covers a broad range of taxa [Table 1]. For a successful transmission the arbovirus is required to

replicate in the arthropod vector. This obligatory replication mainly takes place in the salivary glands of the blood-feeding vector. During a subsequent bloodmeal, the virus is transmitted to a vertebrate.

Table 1: Diversity of Arboviruses (Selected examples).

Arbovirus		Genome	Main Vertebrate Reservoir	Main Vertebrate Host	Main Arthropod Vector Family (Species)
Family *Genus*	Virus Species				
Asfarviridae					
Asfivirus	African Swine Fever virus	dsDNA	Wild pigs	Pigs	*Argasidae* (*Ornithodorus sp.*)
Bunyaviridae					
Nairovirus	*Crimean-Congo haemorrhagic fever nairovirus*	(-)ssRNA	*Ixodidae* (*Haemaphysalis sp., Hyalomma sp.*)	Wild- and lifestock animals	*Ixodidae* (*Haemaphysalis sp., Hyalomma sp.*)
Orthobunyavirus	*Bunyamwera orthobunyavirus*	(-)ssRNA	Ungulates	Ungulates	*Culicidae* *Ceratopogonidae*
Phlebovirus	*Rift Valley fever phlebovirus*	(-)ssRNA	Ungulates	Ungulates	*Culicidae* (*Aedes sp.*, *Culex sp.*, *Mansonia sp.*)
	Sandfly fever Naples phlebovirus	(-)ssRNA	*unknown*	Rodents Humans	*Psychodidae* (*Phlebotomus sp., Lutzomyia sp.*)
Flaviviridae					
Flavivirus	Yellow Fever virus	(+)ssRNA	Primates	Humans	*Culicidae* (*Aedes sp.*, *Haemagogus sp.*, *Sabethes sp.*)
Orthomyxoviridae					
Thogoto virus	Thogoto virus	(-)ssRNA	Rodents	Ungulates	*Ixodidae* (*Rhipicephalus sp.*, *Hyalomma sp.*)
Reoviridae					
Coltivirus	Colorado tick fever virus	dsRNA	Rodents Ungulates	Humans	*Ixodidae* (*Dermacentor sp.*)
Orbivirus	Bluetongue virus	dsRNA	Ungulates	Ungulates	*Ceratopogonidae* (*Culicoides sp.*)
Togaviridae					
Alphavirus	Venezuelan equine encephalitis virus	(+)ssRNA	Rodents	Equines	*Culicidae* (*Culex melanochonion sp.*)

double-stranded DNA (dsDNA), double-stranded RNA (dsRNA), single-stranded RNA (ssRNA),

Arboviruses that actively infect the arthropod vector require the capability to overcome the vector's immune system and they encounter an even more complex immune response in

vertebrates. The obvious need to suppress the host's immune system is achieved through accessory viral proteins that interfere at different steps in the hosts' immune pathway. For example, a nonstructural protein encoded on the S segment (NSs) in bunyaviruses is able to provide such a function [refer to 3.3.1 for coding strategies and to 3.3.3 for functional properties].

Arboviral proteins that inhibit the vector's RNAi pathway have not been identified so far. However, a subgenomic RNA has been discovered in the flavivirus West Nile virus (WNV) that interferes with the RNAi pathway in the mosquito vector. The subgenomic RNA of WNV was observed to bind to a mosquito exonuclease which is required to degrade viral RNA at a downstream step of the RNAi pathway (Moon *et al.*, 2012; Roby *et al.*, 2014).

The evolution of arboviruses is subject to ongoing research in order to understand the evolution of the arboviral dual-host tropism and pathogenicity in vertebrates. Several RNA virus families as well as a single DNA virus family contain arboviruses [Table 1] suggesting that this feature evolved convergently. This dual host tropism seems to be a paraphyletic property because all families containing arboviruses also contain additional taxa with a monotropism for either vertebrates or arthropods. Arboviruses can also be transmitted horizontally and vertically between mosquitoes, which gave rise to the hypothesis that arboviruses might have evolved from insect-specific viruses (Dudas & Obbard, 2015; Elliott, 2014). This hypothesis is underlined by the recent discovery of insect-specific flaviviruses that branch deeper than congeneric arboviruses (Cook *et al.*, 2012). However, the evolutionary origin of arboviruses is still controversially discussed (Nasar *et al.*, 2012).

3.3 Bunyaviruses

The family *Bunyaviridae* is the largest and most diversified family of RNA viruses. The family comprises more than 350 serologically distinct viruses (Plyusnin *et al.*, 2012). Five genera, namely *Hantavirus*, *Nairovirus*, *Orthobunyavirus*, *Phlebovirus*, and *Tospovirus* have been established. To date only one hundred bunyaviruses have been officially classified as distinct bunyavirus species by the International Committee on Taxonomy of Viruses (ICTV) ('Virus Taxonomy: 2015 Release', 2016). The high diversity of taxa within the family is reflected through the high diversity of hosts that can be infected. These range from plants to insects and ticks, to a broad variety of vertebrates, such as rodents, birds, reptiles, ungulates, bats, monkeys, and humans. The family *Bunyaviridae* contains some of the major arboviruses which have a devastating impact on human and animal health. In addition, the non vector-borne hantaviruses cause some of the most severe diseases in humans, such as hemorrhagic fever and influenza-like respiratory illness. The plant-pathogenic tospoviruses cause severe losses in agriculture.

Bunyaviruses are distributed worldwide but appear to have a higher diversity and prevalence in tropical and subtropical regions (Schmaljohn & Nichol, 2007). Several bunyaviruses are considered as emerging and reemerging pathogens due to their recent invasion into new geographic regions and increasing incidence in humans or livestock. Such

as Crimean-Congo hemorrhagic fever virus (CCHFV) in the Balkan pensinsula and Turkey, Rift Valley fever virus (RVFV) in tropical Africa, Sin Nombre virus (SNV) in the Americas, Severe fever with thrombocytopenia virus (SFTSV) in Asia, and Schmallenberg virus (SBV) in Germany (Beer *et al.*, 2013; Bird & Nichol, 2012; Ergonul, 2012; Soldan & González-Scarano, 2005; Watson *et al.*, 2014; Yu *et al.*, 2011).

Orthobunyaviruses, phleboviruses, and nairoviruses are transmitted to their animal or human hosts by blood-feeding arthropods such as mosquitoes, midges, phlebotomine sandflies, and ticks (Elliott, 2014; Plyusnin *et al.*, 2012). The genera *Hantavirus* and *Tospovirus* are unique as the viruses are transmitted by aerosolized rodent excreta (Tsai, 1987) or mechanically by insects (Mandal *et al.*, 2001), respectively. Recently, hantaviruses were also detected in bats and shrews (King et al., 2012; Witkowski *et al.*, 2016).

3.3.1 Virions, Genome and Proteins

Bunyavirus virions are spherical or pleomorph enveloped particles with a diameter of 80–120 nm. The virus particles display glycoprotein projections of about 5–10 nm on their surface. The glycoproteins are embedded in a lipid bilayer which is approximately 5 nm thick and is derived from cellular Golgi membranes, or may stem from cell surface membranes. The genome is single-stranded, negative-sense and trisegmented. Genes for structural and nonstructural proteins can also be encoded in ambisense coding strategies as in phlebo- and tospoviruses (Schmaljohn & Nichol, 2007).

The genome comprises three segments of distinct sizes which are named after their relative length: large (L), medium (M), and small (S) [refer to Table I.2 in Chapter I]. The L segment encodes the viral L protein, an RNA-dependent RNA polymerase (RdRp) which is the largest bunyavirus protein. Bunyamwera virus (BUNV), the prototype of both the *Bunyaviridae* family and the *Orthobunyavirus* genus, has a RdRp protein of 259kDa. The RdRp protein is responsible for the synthesis of mRNA during replication. The M segment encodes a glycoprotein precursor (GPC) protein, which is posttranslationally cleaved into the two surface proteins Gn and Gc. These two proteins are named in accordance to their relative proximity to the amino (Gn) or carboxy terminus (Gc) of the GPC protein. Their function is to fascilitate target cell receptor attachment and entry into the host cell. The GPC protein of BUNV is cleaved into a Gc protein of 110kDa and a Gn protein of 32kDa. Some viruses encode a third cleavage product which is an optional nonstructural protein NSm (18kDa in BUNV). The S segment encodes the viral nucleocapsid (N) protein and an accessory nonstructural protein S (NSs) (26 kDa and 11 kDa respectively in BUNV) (Schmaljohn & Nichol, 2007). The N protein binds the viral genome and forms a complex inside the virion called the ribonucleoprotein (RNP). As a consequence the viral genome is protected by the N protein from recognition by the host cell's immune system (Mir & Panganiban, 2005).

Both accessory nonstructural proteins NSs and NSm are fascilitating viral replication within the host cell. The NSs protein can counteract the immune response of the vertebrate host by inhibiting the induction of interferon (Blakqori *et al.*, 2007; Weber *et al.*, 2002). The NSm protein of RVFV has been shown to be involved in the maturation, replication and

infection of the host cell and it suppresses virus-induced apoptosis [refer to 3.3.3 for functional details] (Gerrard *et al.*, 2007; Won *et al.*, 2007). The NSm protein has not been found in *Hantaviruses* and it is not encoded by all phleboviruses. An NSs protein has not been described for the genus *Nairovirus* and only a few hantaviruses encode this protein (Nichol *et al.*, 2005; Schmaljohn & Nichol, 2007). This indicates that the NSs protein is not consistently represented throughout bunyavirus genera (Elliott, 2000). Members of the serogroups *Anopheles* A, *Anopheles* B, and Tete orthobunyavirus do not encode a NSs protein (Mohamed *et al.*, 2009). This is in contrast to all other orthobunyaviruses and the tick-transmitted phleboviruses. Moreover the insect-borne phleboviruses do not encode a NSm protein but members of the genus *Orthobunyavirus* and the tick-transmitted phleboviruses do encode such a protein (Palacios *et al.*, 2013; Rönnholm & Pettersson, 1987; Yu *et al.*, 2011). NSm and NSs genes are encoded in a genus specific manner [Figure 4]. The NSs gene of members of the genus *Orthobunyavirus* is encoded on the S segment in an overlapping ORF with the N gene. Both proteins are translated from the same mRNA that is complementary to the corresponding virion RNA segment (Eshita *et al.*, 1985). In contrast phlebo- and tospoviruses use an ambisense coding strategy and translate their NSs from a subgenomic mRNA, which has the same polarity as the viral RNA (Ikegami *et al.*, 2005). Similar to orthobunyaviruses, *Hantavirus* NSs is encoded in an overlapping ORF with the N gene. The expression of the NSs protein is enabled by a ribosomal leaky scanning mechanism (Jääskeläinen *et al.*, 2007; Vera-Otarola *et al.*, 2012).

The genome termini of each segment feature short, non-coding nucleotide sequence motifs which are reverse complementary. This enables the genome segments to form panhandle-like structures within the virions. The nucleotide sequences of these motifs vary between genera but are a conserved trait within the family *Bunyaviridae*.

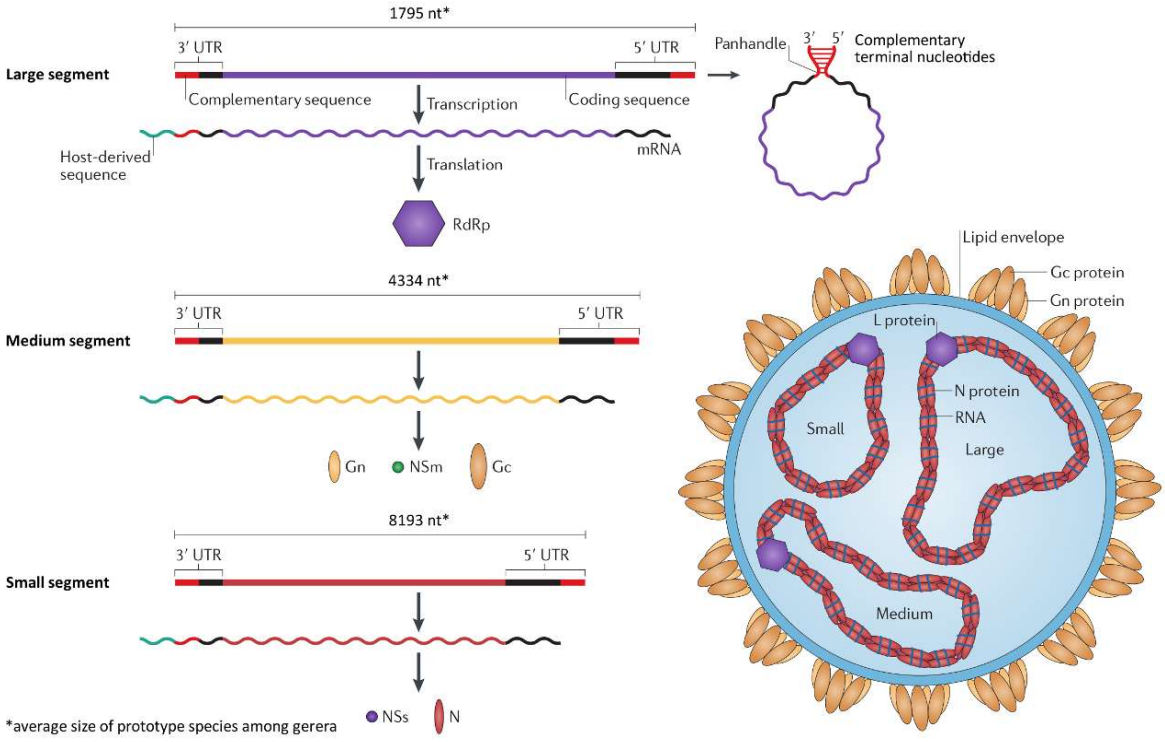

Figure 1. (Legend continued on following page)

Genome organization and virus particle of bunyaviruses. Bunyaviral genome segments are shown as bold horizontal lines. The coding regions of each segment are highlighted in color: L segment in purple, M segment in yellow and S segment in red. The untranslated regions (UTR) are shown in black color at each segment and the terminal complementary sequences are marked in red. The sketch at the top right shows the formation of panhandle-like structures fascilitated by the segment's termini. The viral mRNA produced from each segment are shown in waved lines using the same color code as in the viral genome. Host-derived RNA sequence fragments obtained via cap-snatching mechanism are shown in green at the mRNA's 5' ends respectively. The average segment sizes among prototype species of each bunyavirus genus and symbols for gene products are shown for each segment respectively: RNA-dependent RNA polymerase (RdRp) protein, glycoproteins (Gn and Gc), nonstructural M protein (NSm), nucleocapsid (N) and nonstructural S protein (NSs). The bunyavirus particle to the right shows the arrangement of viral proteins. The ribonucleoprotein (RNP) complexes formed by viral genome RNA, nucleocapsid proteins and a single RdRp protein are shown inside the virus particle in blue, red and purple. Viral glycoproteins Gn and Gc embedded in the lipid envelope of the virus are shown in brown color. (modified after Elliott, 2014)

3.3.2 Replication Cycle

The steps in the viral replication cycle are similar to those of other enveloped viruses. In a first step the virus particles attach to host cell receptors mediated by either one or both of the viral glycoproteins Gn and Gc. The host cell receptors for attachment, internalization and infection of most bunyaviruses are not known. However, β1 and β3 integrins were identified to be used by some hantaviruses to enter host cells (Gavrilovskaya *et al.*, 1999). The receptor binding mediates internalization by endocytosis of the virion/receptor complex into clatherin-coated pits. Formed endocytic vesicles undergo an acidification and as a consequence the Gn and/or Gc protein(s) undergo a conformational change. This facilitates fusion of the viral membrane, with the membrane of the endosome and the release of the viral genome to the host cell cytoplasm. For the phleboviruses RVFV and UUKV a C-type lectin called DC-SIGN expressed on the surface of dendritic cells was shown to bind virus particles (Lozach *et al.*, 2011). The binding is fascilitated via interactions of cellular high-mannose N-glycans with viral glycoproteins.

The primary transcription of the viral mRNAs is catalyzed by the viral RdRp protein. A single RdRp protein is attached to each of the three segments respectively. The first transcription step is primed by host cell-derived primers which are produced by the endonuclease activity of the N-terminal region of the RdRp from host cell mRNAs (cap-snatching mechanism). These primers are about 12-18 nucleotides in length and contain a 5' 7-methylguanylate residue (cap structure) present at host mRNAs. Protected from hydrolyzing enzymes the capped viral mRNA is recognized by the small ribosomal subunit of host cells translation machinery. The viral mRNA translation occurs either at membrane-bound ribosomes are located at the endoplasmatic reticulum (ER) for the M segment or on free ribosomes for the S and L segment, respectively. The M segment's GPC protein is cotranslationally proteolytically cleaved into the structural proteins, Gn and Gc, and (if encoded) a nonstructural protein NSm. It was shown recently that the cleavage of the GPC protein in BUNV is processed by a cellular signal peptidase and a signal peptide peptidase of the host (Shi *et al.*, 2016). Gc and NSm proteins are cleaved at internal signal peptides (SP). The NSm domain I functions as a SP for NSm and NSm domain V as a SP for the Gc protein. A host intramembrane-cleaving protease (signal peptide peptidase) is processing NSm domain I further and is required for cell fusion activities. The NSm domain V as a SP for the Gc protein remains integral to the NSm protein which explains the NSm topology as a two-

membrane-spanning integral membrane protein (Shi et al., 2016). The GPC protein cleavage is followed by a dimerization of Gn and Gc proteins in the ER and subsequent transport to the Golgi complex mediated by a signal in the transmembrane domain in the Gn protein.

The regulation of shifting from primary transcription to replication has not been clarified for any member of the family *Bunyaviridae*. The full-length positive-sense RNA - also known as antigenomic RNA (agRNA) or coding RNA (cRNA), serves as a template for the replication of the genome. This template is produced in a primer independent manner from the negative-sense RNA template - also known as genomic (gRNA) or virion-sense RNA (vRNA). It has been demonstrated, that the M segment is not necessary for genome replication but that a sufficient level of N proteins is required to encapsidate generated +/- RNAs (Dunn *et al.*, 1995). In addition, the N protein was suggested to facilitate the dissociation of the panhandle structures formed by the reverse complementary 3'/5' ends of the viral genome segments and thereby freeing the 3' terminus for RdRp interactions. Furthermore, the N protein is proposed to act as a chaperone by temporary and continuously unfolding RNA (Mir & Panganiban, 2005).

The three negative-sense viral genome segments (gRNA) are converted into positive-sense RNA (agRNA), which are simultaneously encapsidated by the viral N protein and from which virus genome copies (gRNA) are synthesized (Kukkonen *et al.*, 2004). The encapsidation of agRNA is believed to minimize the formation of double stranded RNA in the cell, which is important to avoid detection by the host immune system (Elliott, 2014). For genes encoded in ambisense orientation the agRNA serves as a template for subgenomic RNAs. The RNPs created in this process are then transported to membranes of the Golgi complex. Within these membranes viral Gn and Gc proteins have been inserted and virus particles bud into Golgi membrane-derived vesicles containing the RNPs (Plyusnin & Elliott, 2011). This Golgi apparatus association is typical for bunyaviruses and was shown for the majority of bunaviruses (Kikkert *et al.*, 1999; Plyusnin & Elliott, 2011). However, some members of the genera *Hantavirus* and *Phlebovirus* were found to bud rather at basolateral cellular surface membranes than at the Golgi membrane (Anderson & Smith, 1987; Ravkov *et al.*, 1997). Golgi vesicles that contain fully maturated virus particles are trafficking to the cell surface, which may be facilitated by actin filaments, and the vesicular membranes fuse with the plasma membrane of the cell. As a result infectious virions are released from the cell [Figure 2].

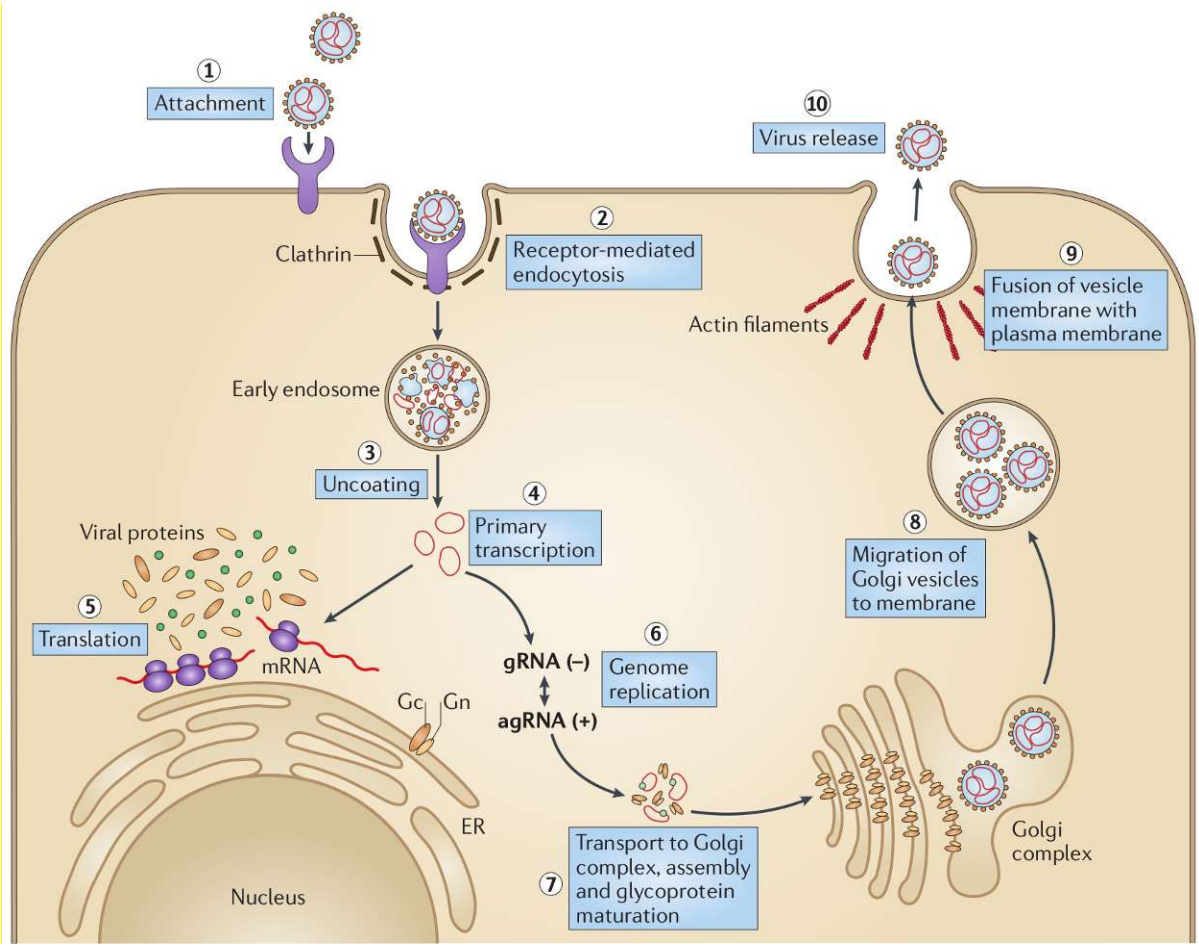

Figure 2. Example of the bunyavirus replication cycle. Virus particles attach to host receptors (1) and get internalized by receptor-mediated endocytosis (2). Endosomes experience an acidification which results in uncoating of the virus particles (3). The viral RNA-dependent RNA polymerase catalyses the primary transcription of viral mRNAs (4) which is followed by translation of the viral mRNAs (5). The three negative-sense viral genomic RNA (gRNA) segments get converted into positive-sense antigenomic RNA (agRNA) (6). The ribonucleoproteins (RNPs) get transported to membranes of the Golgi complex which have been modified by the insertion of Gc and Gn proteins (7). Vesicles containing mature virus particles are trafficking to the cell surface (8) and fuse with plasma membrane (9) which results in release of the virus particles from the cell fascilitated by actin filaments (9). (Elliott 2014, modified)

3.3.3 Immunosuppression and Antiviral Host Response

Viruses as one of the smallest and most minimalistic equipped organisms, depend for their replication on the host cell's machinery. The innate and adaptive immune systems have evolved, to limit (viral) infections in host cells. On the virus side, proteins have evolved that counteract the host immune response in order to fascilitate their replication. Insect viruses like the mosquito-specific entomobirnavirus Culex Y virus (CYV) (Marklewitz *et al.*, 2012) express a viral protein 3 (VP3) that was shown to inhibit the immune system of insects. Briefly, a protein called Dicer binds virus typical replication intermediates of double stranded RNA and cleaves them into small fragments called small interfering RNAs (siRNA). One of the RNA strands is loaded into a protein complex called RISC. This strand serves as a matrix to bind mRNAs by basepairing where as a consequence the matching mRNA is cleaved and destroyed (Nayak *et al.*, 2013). It has been shown that the CYV' VP3 protein suppresses the RNAi pathway by binding long double stranded RNA and therefore protecting it from

recognition by Dicer. Furthermore a direct interference with the Dicer protein was observed. This interaction inhibits Dicer-mediated siRNA production (van Cleef *et al.*, 2014). Similar viral suppressor proteins of the RNAi pathway have convergently evolved in a broad variety of virus families (Obbard *et al.*, 2006). No proteins interfering with the vector's RNAi pathway have been described for arboviruses. A complete suppression of the RNAi pathway as observed in many highly pathogenesic insect-specific viruses, would be detrimental to the viral transmission cycle (Cirimotich *et al.*, 2009; Myles *et al.*, 2008).

In addition to vector's immune system, arboviruses also have to cope with the vertebrate's immune system. As a consequence arboviruses encode proteins that are counteracting the vertebrate host's immune system. The NSs protein of orthobunyaviruses and phleboviruses (family *Bunyaviridae*) was shown to inhibit the cellular interferon response in their vertebrate hosts. The NSs protein of tospoviruses suppress the RNAi pathway in their plant hosts (Bouloy *et al.*, 2001; Bridgen *et al.*, 2001; Soldan *et al.*, 2005; Szemiel *et al.*, 2012; Takeda *et al.*, 2002). The inhibition of the RNAi response by the tospovirus NSs protein was the first RNA silencing suppressor that was identified in negative-strand RNA viruses (Takeda *et al.*, 2002). The NSs protein of the orthobunyavirus La Crosse virus (LACV) has been shown to inhibit the RNAi antiviral activity in mammalian cells (Soldan *et al.*, 2005). In BUNV the NSs protein is required for efficient replication in vertebrates but does not inhibit the RNAi response in the arthropod host (Szemiel *et al.*, 2012). In phleboviruses the NSs protein of SFTSV has been shown to interact with vertebrate proteins responsible for the activation of type I interferon (IFN) responses. The NSs protein binds IFN proteins retinoic acid-inducible gene I (RIG-I), TANK-binding kinase 1 (TBK1) and TRIM25. This complexe is translocated into cytoplasmic endosome-like structures for sequestration and inhibits the expression of IFN as an antiviral response (Qu *et al.*, 2012; Santiago *et al.*, 2014).

The other nonstructural protein encoded by bunyaviruses, NSm, is also involved in suppression of the antiviral host response. It has been shown that the NSm protein of the phlebovirus RVFV is involved in the suppression of virus-induced apoptosis in vertebrates (Won *et al.*, 2007). RVFV NSm inhibits the staurosporine-induced activation of caspase-8 and -9 and suppresses the severity of caspase-3 activation.

The tick-associated nairoviruses are special among bunyaviruses regarding their strategy to counteract the antiviral host response. In contrast to all other bunyaviruses, nairoviruses do not encode a NSs protein. Nariroviruses encode an ovarian tumor (OTU)-like protease motif on the L segment which is encoded at the amino terminus of the L protein. The OTU-like protease has been suggested to suppress the host cell inflammatory and antiviral response and thus plays a role as a pathogenicity factor (Frias-Staheli *et al.*, 2007; Honig *et al.*, 2004; Kinsella *et al.*, 2004). Ubiquitin and the interferon stimulated gene product 15 (ISG15) conjugate to proteins via a conserved C-terminal protein motif (LRLRGG) and mediate important innate antiviral responses. The OTU-like protease of nairoviruses hydrolyzes ubiquitin and ISG15 from cellular target proteins. Therefore it inhibits the NF-κB dependent signaling and antagonizes the antiviral effects of ISG15 (Frias-Staheli *et al.*, 2007).

In summary, the immune system of vertebrates is inhibited by the NSs protein of members of the bunyavirus genera *Phlebovirus* and *Orthobunyavirus*. The NSs protein of plant infecting tospoviruses antagonises the immune response of the plant host. Nairoviruses evolved a different protein which is an OUT-like protease that inhibits the vertebrate hosts immune response. The mechanistic variations and complexity of host immune response modulating proteins in bunyaviruses is remarkable and it underlines the extraordinary diversity of this virus family.

3.3.4 Taxonomic Classification

Since 1971 the responsibility to classify viruses and select suitable criteria for virus classification is assigned to the ICTV. To date the family *Bunyaviridae* has not been assigned to a taxonomic order. The closest phylogenetically related virus families are *Arenaviridae* and *Orthomyxoviridae* (King *et al.*, 2012).

Since 2002 the family *Bunyaviridae* is subdivided into five distinct genera namely *Hanta-*, *Nairo-*, *Orthobunya-*, *Phlebo-*, and *Tospovirus* as described above [refer to 3.3]. To date no mutual criteria for bunyavirus species classification and genus assignment have been established. Genus specific characteristics are inconsistently taken into account for bunyavirus classification: virion morphology, conserved consensus terminal nucleotides of genome segments, coding strategy of nonstructural and structural proteins, serological cross reactivity as well as the transmission mechanism (Plyusnin *et al.*, 2012). Historically the serological relationship among bunyaviruses played the most important role for taxonomic classification of the establishment of the Bunyamwera supergroup of viruses (Elliott, 2014). After further biochemical and structural information was taken into account the family *Bunyaviridae* was established in 1975 (ICTV, 1975). Serological cross reactivity is still important for taxonomic classification of bunyaviruses but virus isolates and respective antibodies are not always available (Schmaljohn & Nichol, 2007). With the advent of sequencing techniques genetic and patristic distance criteria become nowadays more relevant in virus classification. For example, members of the genus *Orthobunyavirus* are discriminated as separate species if the N protein sequences differ by more than 10% (Plyusnin *et al.*, 2012). Phylogenetic analyses showed that genetic distances are in line with serological-based classification (King *et al.*, 2012). Genetic-based classification criteria have already been implemented in other families where viruses are difficult to isolate, e.g. family *Coronaviridae* (de Groot *et al.*, 2012).

Three established genera named *Emara-*, *Phasma-* and *Tenuivirus* that have not been assigned to a virus family group within the phylogenetic diversity of the family *Bunyaviridae* and are described below (Ballinger *et al.*, 2014; Tatinenia *et al.*, 2014; Toriyama *et al.*, 1998).

3.3.4.1 Emaraviruses

The unassigned genus *Emaravirus* with its eponymous type species European mountain ash ringspot-associated virus (EMARAV) shares similarities with members of the family *Bunyaviridae* as well as with the unassigned genus *Tenuivirus*. Bunyavirues and emaraviruses have spherical enveloped virions with a diameter of about 80-100 nm, a segmented genome with negative polarity and complementary 5' and 3' genome segment termini (King *et al.*, 2012). Apart from the prototype EMARaV, four other members are assigned to the genus: Fig mosaic-, Pigeonpea sterility mosaic-, Raspberry leaf blotch-, and Rose rosette virus ('Virus Taxonomy: 2015 Release', 2016). The RdRp proteins of emaraviruses show closest relationship to the bunyavirus genera *Tospovirus* and *Orthobunyavirus*. As a commonality tospoviruses and emaraviruses infect plants. Emaravirues have been suggested to be transmitted by mites (Seifers *et al.*, 1997; Skare *et al.*, 2006). The 3' and 5' ends of *Emaravirus* RNA genome segments are complementary and their conserved sequence resembles those of the genus *Orthobunyavirus*. Despite theses commonalities, emaraviruses can have up to threefold more genome segments than bunyaviruses. For example, the genome of Wheat mosaic virus (WMV) comprises eight RNA segments and represents the largest emaravirus genome known so far (Tatinenia *et al.*, 2014). Nevertheless the major viral proteins, like the RdRp protein, the GPC protein (that is posttranslationally cleaved into the glycoproteins Gc and Gn) as well as the N protein are encoded in a bunyavirus-like manner on the three largest segements in all emaraviruses (Mielke-Ehret & Mühlbach, 2012; Mühlbach & Mielke-Ehret, 2012; Tatinenia *et al.*, 2014). The additional smaller genome segments may have been acquired during the diversification of emaraviruses. The inclusion of the genus *Emaravirus* into the family *Bunyaviridae* is currently under discussion.

3.3.4.2 Phasmaviruses

The first members of the proposed new genus *Phasmavirus* were discovered only recently (Ballinger *et al.*, 2014). Ballinger and coworkers identified two novel viruses in larvae of phantom midges (*Chaoborus sp.*) collected from polar freshwater ponds at the tundra-boreal boundary of North America (Ballinger *et al.*, 2014). The viruses were named Kigluaik phantom virus (KIGV) and Nome phantom virus (NOMV). KIGV and NOMV could not be isolated in cell culture. An increase of KIGV genome copies was documented after primary infection in *Aedes albopictus* (C6/36) cells and a weak increase in genome copies could be observed after a two-week passage. Further passages were not successful, leaving doubts whether the viruses were isolated or not. The group performed RT-PCR assays to confirm the existance of viral exogenous RNA and to exclude expressed endogenous RNA copies with a bunyavirid-like sequence (Ballinger *et al.*, 2014). Like bunyaviruses, the genome of phasmaviruses is comprised of three ssRNA segments (S, M and L) with negative polarity. Coding strategies of phasmaviruses seem to be similar to bunyaviruses. The gene encoded on the L segment shows 30% similarity to the RdRp gene of members of the family *Bunyaviridae*. The translated RdRp genes of phasmaviruses contain the highly conserved

bunyavirus RdRp gene motifs like the SDD aa motif at the nucleotide addition site of motif C [Table 3]. The M segment shows similarities to the GPC protein of bunyaviruses and the putative cleavage site $_{255}$ILG/CDN is similar as in phleboviruses. The S segment is likely to encode an N protein. Conserved terminal nucleotides as observed in bunyaviruses are present in all genome segments [Table 2]. The nucleotide sequence of the terminal nucleotides of phasmaviruses is unique and matches none of the established genera of the family *Bunyaviridae* nor tenui- or emaraviruses. Phylogenetic analysis placed phasmaviruses in sister relationship to the superclade of the bunyavirus genera *Hanta-*, *Orthobunya-* and *Tospovirus* suggesting that phasmaviruses are members of the family *Bunyaviridae* (Ballinger *et al.*, 2014).

3.3.4.3 Tenuiviruses

Rice stripe virus (RSV) causes a disease in rice known since 1931 and is the prototype species of genus *Tenuivirus* (Kuribayashi, 1931a). As a pecularity tenuiviruses apparently seem to exist as free RNPs without an envelope. The RNPS appear to be spiral-shaped, branched or circular (King *et al.*, 2012). Tenuiviruses and bunyaviruses share the feature of a segmented, single stranded RNA genome with reverse complement terminal nucleotides (King *et al.*, 2012). However, the genome of tenuiviruses consists of four to six segments which is distinct from members of the family *Bunyaviridae* having a tripartite genome (Shirako *et al.*, 2012). The second largest segment (RNA2) in tenuiviruses encodes a nonstructural protein that has been shown to accumulate in plants but its function remains unknown. Like bunyaviruses, the tenuivirus' genome encodes an RdRp gene on the largest segment (RNA1) and the N gene on the third largest segment (RNA3). Like arenaviruses and all members of the genera *Phlebovirus* and *Tospovirus*, members of the genus *Tenuivirus* have an ambisense coding arrangement and as in all viruses having a negative-sense segmented genome, virus replication involves an mRNA generation using a cap-snatching mechanism (Nguyen *et al.*, 1997). Tenuiviruses share with phleboviruses eight conserved nucleotides at their genome termini. Tospoviruses and members of the unassigned genus *Tenuivirus* are both infecting plants and are transmitted by an arthropod vector. Tenuiviruses are mechanically transmitted by planthoppers like *Laodelphax sp.*, *Unkanodes sp.*, and *Terthron sp.* (Hirao, 1968; Kuribayashi, 1931a, b).

Toriyama and coworkers proposed that Rice grassy stunt virus (RGSV) can be classified as a separate genus (Toriyama *et al.*, 1998). RGSV has six RNA segments that encode four or five additional proteins to those ones that are classically encoded by a tenuivirus genome. Furthermore, sequence similarities of the RGSV proteins with those of other tenuiviruses are very low (Toriyama *et al.*, 1998).

3.4 Virus Diversification and Evolution

The high mutation rate of viruses and the influence of environmental factors is driving both the evolution of the virus and the evolution of their hosts [refer to 3.4.1]. Like all negative-sense RNA viruses the bunyavirus RdRp protein is lacking a proof-reading activity and a considerable amount of genetic heterogenicity within a virus population (also known as the quasispecies cloud) is generated during replication (Elliott, 2014; Nowak, 1992). A bottleneck situation can favour a certain genetic virus variant within a virus population. As a consequence, this variant can emerge as the dominating one after the recovery of the bottleneck situation (Forrester *et al.*, 2012).

Viruses with a segmented genome, like bunyaviruses, can exchange complete genome segemtns with viruses of the same species (a mechanism named reassortment) and thereby experience drastic genetic changes. For example, reassortant genotypes of influenza viruses show a higher virulence, like Influenza A H1N1 that caused an outbreak in 2009 (known as the swine flu) or Influenza A H5N1 (known as the bird flu) that is highly pathogenic for birds (Cohen, 2009; Liu, 2005).

During co-infection of a single cell with two different bunyaviruses, reassortment can potentially generate six reassortant viruses due to their tripartite genome (Elliott, 2014). However, there seem to be limitations to reassortment such as incompatibility of certain genome segment combinations and a restriction to closely related taxa (Iroegbu & Pringle, 1981). The molecular background for this restriction has not yet been revealed, but this presumably involves interactions between the N protein, the RdRp proteinand the viral RNA (Elliott, 2014). For bunyaviruses, especially in the genus *Orthobunyavirus*, reassortant viruses were intensively studied revealing a higher tendency to aquire a new M segment. The M segment encodes the gene products (Gn, Gc and NSm proteins) that are responsible for receptor binding and it thereby determines which arthropod species can be infected (Beaty *et al.*, 1981). Reassortant viruses can acquire new properties such as the acquisition of a new arthropod vector which might have different feeding preferences and can guide the reassortant virus to a potential new host.

Like described above for influenza viruses, an increase in pathogenicity has also been described for bunyavirus reassortants. Reassortants may occur within a virus species: The genome of the *Bunyamwera orthobunyavirus* reassortant member Ngari virus (NRIV) comprises the L and S segment of the bunyavirus prototype BUNV and the M segment of Batai virus (BATV), another member of the species *Bunyamwera orthobunyavirus* (Briese *et al.*, 2006). Both viruses, BUNV and BATV, infect humans and cause a relatively mild febrile illness. In contrast, NRIV is associated with severe haemorrhagic fever. Another example is Garissa virus, a reassortant with L and S segment of BUNV and the M segment originating from an unknown bunyavirus. Garissa virus has a higher pathogenicity than BUNV and an infection can cause a severe haemorrhagic disease (Bowen *et al.*, 2001). In contrast, BUNV causes a mild febrile illness with headache, arthralgia and rash (Gonzalez & Georges, 1988). Recently SBV was identified to be associated with abortions and malformations in ungulates. SBV belongs to the species Sathuperi virus and is a possible ancestor of the reassortant

Shamonda virus which is containing the S and L segment of SBV and the M segment from an unclassified virus (Goller *et al.*, 2012).

Arboviruses experience dual selection pressure during their infection of a vertebrate host and of an arthropod vector. On the other hand insect viruses, having a monotropism, are only subject to a one-sided selection pressure. A virus with dual host tropism may have to pass more complex barriers than viruses having a monotropism. Assuming that evolution may lead to more complex structures over time, a host monotropism may be more ancient than a dual host tropism.

3.4.1 Endogenous Virus Elements and the Origin of Viruses

Viruses are believed to be ancient organisms, either emerging before the formation of the first cells or during the ensuing period (Koonin *et al.*, 2015; Luria & Darnell, 1978). A long time of virus-host coevolution which shaped both the viruses and their hosts has been suggested. As discussed by Calisher and Tesh there is a significant difference between the discovery of a 'virus' and the detection of a 'nucleic acid sequence' (Calisher & Tesh, 2014). The molecular detection of a nucleic acid is not consequently equivalent to the detection of a live-virus. Further characteristics, primarily the ability to replicate in a host cell, should be confirmed. This should be considered as a dogma for virologists because of the rising numbers in publications identifying viral sequences as part of a host genome (Ballinger *et al.*, 2013; Li *et al.*, 2015). Integration into host genomes has been shown for a broad variety of vertebrate (Horie *et al.*, 2010; Katzourakis & Gifford, 2010) and invertebrate taxa (Crochu *et al.*, 2004). These viral sequences are called endogeneous viral elements (EVE) and some authors refer to them as Paleoviruses (Patel *et al.*, 2011). Endogenous retroviruses are a well-known class of EVE's and ensure as part of their life cycle an interation into the host genome by providing their own reverse transcriptase. A second class of EVE's are nonretroviral integrated RNA viruses lacking their own reverse transcriptase but having a DNA stage during their replication. Bunyaviruses may also integrate into genomes their hosts during coevolution, a fact that has to be considered in the evaluation of results describing the detection of viral nucleic acids with similarities to bunyaviruses. Ballinger and coworkers detected phlebovirus-like sequence fragments in the genome of a *Daphnia pulex* species complex (Ballinger *et al.*, 2013). Their analysis revealed that the association between phleboviruses and *Daphnia* is at least as old as the common ancestor of the species complex (Ballinger *et al.*, 2013). Following this hypothesis the group identified virus-like sequences in the genomes of other insects like *Drosophila* sp. and *Anopheles* sp. and determined the age for this virus group of 20 million years. Haematophagous insects are mainly found in the sub-order *Nematocera* which includes apart from haematophagous insects like black flies (*Simuliidae*), sandflies (*Phlebotominae*), mosquitoes (*Culicidae*) and gnats (*Ceratopogonidae*) several families with non-haematophagous members. Bunyaviruses are found in the families and subfamilies *Culicidae*, *Phlebotominae* and *Chaoberidae* (Auguste *et al.*, 2014; Ballinger *et al.*, 2014). All members of the sub-order share a larval stage in water and some of them prey

on other larval stages from the same sub-order. For example the larvae of *Chaoborus* flies are one of the most important predators on mosquito larvae and pupae as well as on *Daphnia sp.* as a *Crustaceae* species. Some families within the Nematocera also share special habitats such as occurrence in caves of moth flies (*Psychodidae*), fungus gnats (*Mycetophilidae* and *Keroplatidae*), crane flies (*Tipulidae*) and winter crane flies (*Trichoceridae*). These overlaps in the biology of bunyavirus carrying species are just a selection of opportunities for individuals getting into contact with potentially virus-infected members of other species. Further research should be performed on both the validation of certain taxa actually being infected and to trace the evolution of *Bunyaviridae* across host phylogeny across the sub-order Nematocera.

The origin of insects has been dated to the early Ordovician at approximately 479 million years ago and Crustaceans like the genus *Daphnia* emerged even earlier (530 million years ago) during the evolution of life (Misof *et al.*, 2014). Further research revealed (bunya)virus-like sequences (RdRp, N) in other Crustacean genomes like the common pill-bug *Armadillum vulgare*. Virus-like sequences with relationship to *Mononegavirales*, *Totiviridae*, *Circoviridae* and *Parvoviridae* were detected (Theze *et al.*, 2014). In addition to bunyavirus-like sequences in *Armadillum vulgare*, Metegnier and coworkers detected EVEs in other *Armadillum* species (*Armadillum nasatum*) furthermore in two *Daphnia* species *Daphnia pulex* and *Daphnia pulicaria* as well as in the genome of a sea louse, a parasite of salmons called *Lepeophtheirus salmonis*. In these species the EVEs consist of the bunyavirus N and RdRp gene and in addition *Circoviridae*, *Parvoviridae*, *Totiviridae* & *Mononegavirales*-like sequences (Metegnier *et al.*, 2015). Bunyavirus sequences, more precisely sequences with homology to the unassigned genus *Phasmavirus*, have been detected in six orders of the class Insecta: *Coleoptera*, *Diptera*, *Hemiptera*, *Hymenoptera*, *Lepidoptera* and *Strepsiptera* (Ballinger *et al.*, 2014). The widespread association of (bunya)virus-like sequences with the genomes of a broad variety of ancient animal groups is pointing in the direction of a very long coevolution of viruses and their hosts. However, it underlines the importance to varify if detected nucleic sequences belong to live-viruses or if the sequences have integrated into the insect genome. The basal phylogenetic position of insect-associated viruses and viral sequences obtained from arthropod genomes rised the hypothesis of an arthropod origin of taxonomic virus groups having members with a vertebrate tropism (Dudas & Obbard, 2015; Shi *et al.*, 2015).

4. AIMS OF THE THESIS

To date virus diversity is dominated by taxa identified as a cause of a disease. This obviously leads to a detection bias for pathogenic over nonpathogenic viruses (Cook *et al.*, 2013; Jaenike, 2012; Junglen & Drosten, 2013; Longdon & Jiggins, 2012). Therefore, in particular the diversity of arthropod-specific viruses may be underestimated and it could be even higher than the virus diversity in vertebrates. Thus, especially for RNA viruses, a great genetic diversity is suggested to remain undetected so far, as we lack reliable detection systems to identify taxa distantly related to pathogenic viruses.

Six previously unknown viruses were isolated in insect cull culture from tropical mosquitoes during previous studies. Short genome fragments suggested the detection of novel viruses with distant relationship to bunyaviruses.

The aim of this thesis was the phenotypic and molecular characterization of the six viruses. Specifically, this included to determine virus particle morphologies, to sequence and analyse the entire genomes and to identify replication strategies, as well as expressed proteins. The phylogentic relationship of the viruses should be resolved to provide a tentative taxonomic classification. The host range of the novel viruses should be analysed by infection experiments in cell culture and by serology.

Chapter I

Gouléako Virus Isolated from West African Mosquitoes Constitutes a Proposed Novel Genus in the Family *Bunyaviridae*

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I.1 INTRODUCTION

The family *Bunyaviridae* comprises the five genera *Hantavirus*, *Nairovirus*, *Orthobunyavirus*, *Phlebovirus*, and *Tospovirus* (Nichol *et al.*, 2005). Classification was originally based on serological relationships but has been extended to include virion morphology, genome organization, and phylogenetic relationships. Congeneric members have further features in common, such as conserved genome termini, identical coding strategies, and encoded proteins (Nichol *et al.*, 2005). The segmented, negative-stranded RNA genome codes for a N protein, two glycoproteins (Gn and Gc), and an RdRp on the S, M, and L segments, respectively. S and M segments of the genera *Orthobunyavirus*, *Phlebovirus*, and *Tospovirus* encode two additional nonstructural proteins, NSs and NSm. However, these proteins are not consistently represented throughout those genera (Elliott, 2000; Mohamed *et al.*, 2009). More recently identified novel bunyaviruses, as well as recently sequenced bunyaviruses from archived material, consistently belonged to any of the five known genera (Collao *et al.*, 2009; Elliott, 2000; Lambert & Lanciotti, 2009; Liu, 2003; Major *et al.*, 2009; Moureau *et al.*, 2010; Xu *et al.*, 2007; Yandoko *et al.*, 2007; Yu *et al.*, 2011; Zhioua *et al.*, 2010).

I.2 RESULTS

During an arbovirus surveillance study in Côte d'Ivoire, an RdRp fragment of a novel bunyavirus was identified (Junglen *et al.*, 2009a). The virus was detected with a relatively high prevalence of 6.5% in different species of *Anopheles*, *Culex*, and *Uranotaenia* mosquitoes in a diverse range of habitat types, indicating a widespread virus that is promiscuous regarding arthropod vectors. The virus was tentatively termed Gouléako virus (GOLV), after the village from which the first isolate originated. Here we determined the complete genome sequence and investigated criteria to formally classify GOLV.

Virus isolation from 432 pools of 4.839 female mosquitoes was done in *Aedes albopictus* (C6/36) cells as described before (Junglen *et al.*, 2009b). All positive pools induced similar cytopathic effects (CPE) after 3 to 7 days postinfection (dpi), and maximum genome copies were reached after 5 dpi [Figure I.1a, b, and d]. Polymorphic, enveloped virions with a bunyavirus-like morphology were detected by electron microscopy in infected cell culture supernatants [Figure I.1c] (Junglen *et al.*, 2009b; Quan *et al.*, 2010).

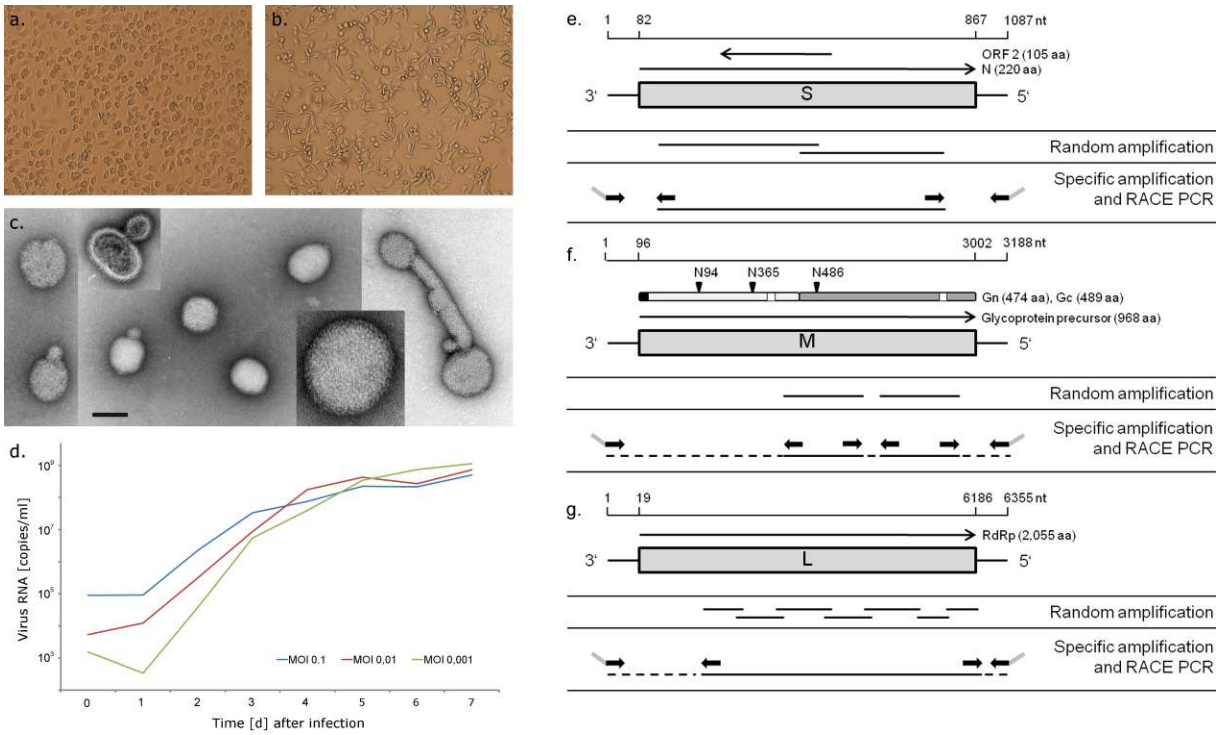

Fig. I.1. GOLV growth on insect cells, morphology, and genome characteristics. (a) Uninfected C6/36 cells. (b) C6/36 cells 4 days after infection with GOLV. (c) Negative staining electron microscopy of purified GOLV particles. Bar = 100 nm. (d) Numbers of GOLV genome copies per ml in cell culture supernatant of C6/36 cells infected with GOLV at MOIs of 0.1, 0.01, and 0.001 were measured by RT-PCR for 7 days. (e to g) Strategies used for full genome sequencing. The top panel shows the genome segments S (e), M (f), and L (g). Boxes represent open reading frames (ORFs) flanked by noncoding regions (NCR), which are indicated by lines. Coding directions are indicated as arrows. Glycoprotein precursor properties were identified by signalP-NN, TMHMM, and NetNGlyc 1.0 and are marked as follows: signal peptide, black box; Gn, light-gray box; Gc, dark-gray box; transmembrane domains (TMD), white boxes; and glycosylation sites, black triangles. Bars in the middle panel indicate genome fragments generated in initial random amplification reactions. The bottom panel shows specific PCRs used for genome walking. Oligonucleotide orientations and positions are marked by arrowheads.

To investigate the GOLV cell tropism, infectious cell culture supernatant of one isolate (A5/CI/2004) was used to infect various insect, reptile, bird, and mammalian cells with multiplicities of infection (MOIs) of 10, 1, 0.5, and 0.1 (measured by 50% tissue culture infective dose [$TCID_{50}$]), and cells were incubated at 33°C and 37°C [Table I.1]. Cell culture supernatants were passaged in fresh cells every 7 days in 1/10 dilutions for five consecutive passages and tested by real-time reverse transcription-PCR (RT-PCR) (Junglen *et al.*, 2009a). GOLV replicated well on U4.4 cells, but on all other cell lines tested, no CPE was observed, and no virus growth was detected. Furthermore, 269 pools of 1.716 adult male mosquitoes were tested for GOLV by real-time RT-PCR, yielding two positive pools of *Culex* spp. and *Anopheles* spp.

Table I.1. Cell lines inoculated with GOLV

Cell line	Host	Tissue	Comment	GOLV growth
C6/36	*Aedes albopictus*	Larvae	RNAi deficient*	Positive
CEF	*Gallus gallus domesticus*	Embryo	Primary cells	Negative
BHK-21	*Mesocricetus auratus*	Kidney		Negative
BHK-J	*Mesocricetus auratus*	Kidney		Negative
EiNi/41	*Eidolon helvum*	Kidney		Negative
ICR-2A	*Rana pipiens*	Embryo		Negative
L929	*Mus musculus*	Fibroblasts		Negative
MEF MDA5$^{-/-}$	*Mus musculus*	Fibroblasts	MDA5 knockout	Negative
MEF RIG-I$^{-/-}$	*Mus musculus*	Fibroblasts	RIG-I knockout	Negative
PSEK	*Sus scrofa domestica*	Kidney		Negative
RoNi/7-NPro.1	*Rousettus aegyptiacus*	Kidney		Negative
S2	*Drosophila melanogaster*	Larvae		Negative
U4.4	*Aedes albopictus*	Larvae		Positive
Vero B4	*Cercopithecus aethiops*	Kidney		Negative
VeroE6/7	*Cercopithecus aethiops*	Kidney		Negative
VH2	*Daboia russelii*	Heart		Negative

*RNAi, RNA interference.

The third passage in C6/36 cells of isolate A5/CI/2004 was completely sequenced. Initial sequences were obtained by adaptor-based random amplification [Figure I.1e to g] (Junglen *et al.*, 2009b; Stang *et al.*, 2005). Two hundred eighty-one clones with inserts between 500 and 1.500 nucleotides (nt) were sequenced and compared to GenBank sequences, showing distant relationships on the amino acid (aa) level with members of the genus *Phlebovirus*. Larger contiguous sequence fragments of 5.275 nt (corresponding to the L gene), 1.049 nt (S gene), and 762 nt (M gene, two fragments) were assembled. M gene fragments were combined into a 1.776-nt fragment. Lateral parts of genome segments were amplified with contig-specific primers and oligonucleotides priming conserved panhandle elements of phleboviruses ligated to an anchor sequence. Genome termini were determined by rapid amplification of cDNA ends-PCR (RACE-PCR). The complete genome was resequenced for confirmation on both strands by primer walking techniques. S-segment size was confirmed by RACE-PCR with virus obtained from cells infected with an MOI of 0.001 and harvested at 3 dpi to avoid detection of defective interfering (DI) RNAs. The GOLV genome was shorter than that of any known member of the *Bunyaviridae* and most similar to those of members of the genus *Phlebovirus* [Table I.2] (Rönnholm & Pettersson, 1987). The genome termini of GOLV were most similar to those in the genus *Phlebovirus*, albeit S and M genome termini were shorter, with a length of only 5 instead of 8 nt [Table I.2]. Notably, a novel phlebovirus, SFTSV, was recently identified in patients in China (Yu *et al.*, 2011). SFTSV also had shorter genome termini, of only 5 instead of 8 nt, in its M and L segments [Table I.2]. The genome termini are generally conserved within but invariably different between bunyavirus genera (Nichol *et al.*, 2005).

Table I.2. Terminal nucleotide sequences and segment sizes of GOLV compared to those of members of other genera in the family *Bunyaviridae*

Genus/ Virus	Consensus terminal nucleotides*		Segment size, in nucleotides (GenBank accession no.)		
			S	M	L
Hantavirus/ Hantaan virus	3' AUCAUCAUCUG–		1.696 (M14626)	3.616 (M14627)	6.533 (X55901)
	5' UAGUAGUAUGC–				
Orthobunyavirus/ Bunyamwera virus	3' UCAUCACAUG–		961 (D00353)	4.458 (M11852)	6.875 (X14383)
	5' AGUAGUGUGC–				
Nairovirus/ Dugbe virus	3' AGAGUUUCU–		1.712 (M25150)	4.888 (M94133)	12.255 (U15018)
	5' UCUCAAAGA–				
Tospovirus/ Tomato spotted wilt virus	3' UCUCGUUA–		2.916 (D00645)	4.821 (S48091)	8.897 (D10066)
	5' AGAGCAAU–				
Phlebovirus/ Rift Valley fever virus	3' **UGUGUUUC**–		1.690 (X53771)	3.885 (M11157)	6.404 (X56464)
	5' **ACACAAAG**–				
Phlebovirus/ Uukuniemi virus	3' **UGUGUUUC**–		1.720 (M33551)	3.229 (M17417)	6.423 (D10759)
	5' **ACACAAAG**–				
Phlebovirus/ Severe fever with thrombocytopenia virus	S segment.	3' **UGUGUUUC**–	1.744 (HM745930)	3.378 (HM745931)	6.368 (HM745932)
		5' **ACACAAAG**–			
	M segment.	3' **UGUGU**UUC–			
		5' **ACACA**GAG–			
	L segment.	3' **UGUGU**UUC–			
		5' **ACACA**GAG–			
Unassigned/ Gouléako virus	S segment.	3' **UGUGU**UUC–	1.087 (HQ541736)	3.188 (HQ541737)	6.358 (HQ541738)
		5' **ACACA**GUG–			
	M segment.	3' **UGUGU**UUC–			
		5' **ACACA**GUG–			
	L segment.	3' **UGUGUUUC**–			
		5' **ACACAAAG**–			

*Boldface type indicates consensus terminal nucleotides between phleboviruses, SFTSV, and Gouléako virus.

In deduced amino acid sequences, the highest L-segment similarity was identified with Uukuniemi virus (UUKV) (28%), SFTSV (27%), and RVFV (27%). The conserved motif III of the RdRp gene was most similar to that of members of the genus *Phlebovirus* [Figure I.2a] (Aquino *et al.*, 2003; Müller *et al.*, 1994). However, there were clear differences discriminating GOLV from all other bunyaviruses. Notably, between the two invariant

residues KW in the tentative RdRp motif A, GOLV showed a V insertion. This insertion was unique among RdRp of negative- strand viruses (Poch *et al.*, 1990). Valine is an uncharged residue and performs due to its dipolar compounds as zwitterion. An insertion of cysteine or tyrosine, also zwitterions, into polymerase active sites has been reported in retroviruses and retrotransposons (Poch *et al.*, 1990).

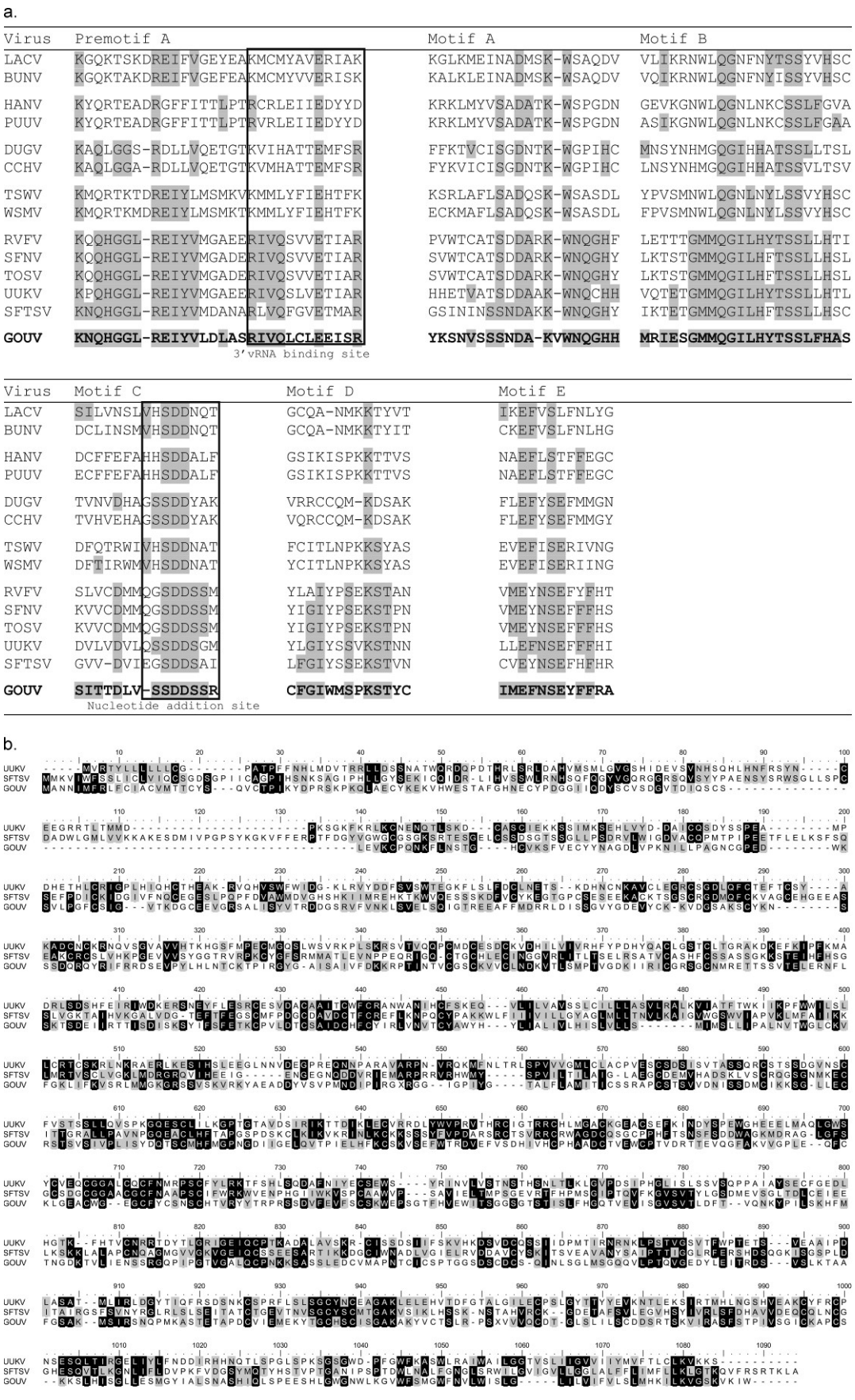

Figure I.2. Multiple sequence alignments of putative GOLV RNA-dependent RNA polymerase and glycoprotein precursor genes. (a) Alignment of GOLV and RdRp genes, third conserved motif. Premotif A and motifs A, B, C, D, and E are indicated. Amino acids conserved

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between GOLV and other bunyaviruses are marked in gray. Active sites corresponding to the PB1 protein of influenza virus (Aquino *et al.*, 2003) are highlighted by boxes. (b) Alignment of putative GOLV, UUKV, and SFTSV glycoprotein precursor proteins. Highly conserved amino residues are marked in black and conserved residues in gray. Abbreviations: BUNV, Bunyamwera orthobunyavirus; CCHV, Crimean-Congo hemorrhagic fever nairovirus; DUGV, Dugbe hantavirus; GOLV, Gouléako virus; HANV, Hantaan hantavirus; LACV, La Crosse orthobunyavirus; PUUV, Puumala hantavirus; RVFV, Rift Valley fever phlebovirus; SFNV, sandfly fever Naples phlebovirus; SFTSV, SFTS phlebovirus; TOSV, Toscana phlebovirus; TSWV, tomato spotted wilt tospovirus; UUKV, Uukuniemi phlebovirus; WSMV, watermelon silver mottle tospovirus.

The M segment was distantly related to the glycoproteins of SFTSV (24%), UUKV (21%), and Punta Toro virus (PTV) (21%). Three in-frame translation initiation codons (AUG) at genomic positions 96, 111, and 141 were found. The first AUG seemed to be in best Kozak context for initiation of translation (Kozak, 1986, 1991). The mosquito-borne RVFV and the sandfly-borne PTV have 5 and 13 in-frame AUG codons, respectively, while the tick-borne UUKV has only one (Gerrard *et al.*, 2007; Kakach *et al.*, 1989; Matsuoka *et al.*, 1988; Rönnholm & Pettersson, 1987) and SFTSV has two. As GOLV is in basal phylogenetic position to a clade formed by the *Phlebovirus* main group (sandfly fever group [SFG]), UUKV, and SFTSV, functional start codons may have been acquired convergently in SFG and GOLV or lost in UUKV and SFTSV. Translation of different proteins from alternative AUG codons has been reported for RVFV (Kakach *et al.*, 1989). However, the function of multiple AUG codons is still unclear.

The most likely cleavage site of the Golgi retention and targeting signal was found between aa 21 and 22 (CYS-QV) [Figure I.1f]. Conserved domains of the phleboviral G1 superfamily (pfam07243) and phleboviral G2 superfamily (pfam07245) were detected by alignment to the pfam database, suggesting Gn to be encoded from aa 1 to 474 and Gc from aa 479 to 968. These coding regions could be confirmed by aligning the GOLV glycoprotein precursor sequence with those from representative phleboviruses. The putative cleavage site between Gn and Gc was identified at aa 479 (CSSRA/TP-CSTSVV, with amino acids conserved among GOLV and phleboviruses underlined) [Figure I.1f and Figure I.2b]. It should be mentioned that SFTSV does not contain the conserved CS motif. Determination of the hydropathy profile predicted two transmembrane domains at aa 370 to 392 and aa 932 to 954, suggesting type I transmembrane topologies for Gn and Gc [Figure I.1f]. N-linked glycosylation sites are conserved within bunyavirus genera except for the genus *Phlebovirus*, where UUKV represents an exception. Members of the SFG contain one N-linked glycosylation site in NSm, one in Gn, and four in Gc, whereas UUKV contains four sites in both glycoproteins (Kakach *et al.*, 1989; Rönnholm & Pettersson, 1987) and SFTSV contains two sites in Gn. The distinct predicted glycosylation pattern in GOLV is another criterion of its distinction from phleboviruses [Figure I.1f]. Contrary to the SFG but in agreement with UUKV and SFTSV, no NSm protein was identified for GOLV, based on sequence alignments and homology searches (Elliott, 2000; Rönnholm & Pettersson, 1987; Yu *et al.*, 2011) [Figure I.2b].

Pairwise comparison of the S segment revealed equally low maximal amino acid similarities with the N protein genes of sandfly fever Sicilian virus (27%), RVFV (27%), and SFTSV (25%). Four ORFs in reverse orientation overlapped the putative N ORF; three seemed too small to encode relevant proteins (all were <70 aa), but one ORF might encode a putative uncharacterized protein of 11.6 kDa [Figure I.1e], a predicted molecular mass

similar to that of the NSs protein of orthobunyaviruses. However, in orthobunyaviruses NSs is encoded within the N ORF in the same coding direction. In phlebo- and tospoviruses, NSs is between 29 and 52 kDa and is encoded in ambisense in a nonoverlapping ORF separated from N by an RNA hairpin fold (Giorgi *et al.*, 1991; Simons *et al.*, 1990). Downstream of N, GOLV contained a small ORF of 38 aa (3.9 kDa) in ambisense orientation with a putative intergenic region of 61 nt and 63.9% A-T content, comparable to that of UUKV (74 nt, 62% A+T content) and SFTSV (55 nt, 67% A+T content) (Simons *et al.*, 1990). By use of mfold, hairpin structures were predicted for the GOLV region downstream of N up to the 5' terminus, suggesting a function in the regulation of transcription as assumed for viruses using an ambisense coding strategy (Schmaljohn & Nichol, 2007; Zuker, 2003). No putative NSs ORF using a coding strategy similar to that for other bunyaviruses could be identified. However, whether ORF2 is expressed and might serve functions similar to those of bunyaviral NSs proteins remain to be determined.

In the phlebovirus RVFV, the NSs and NSm proteins are dispensable for replication in cell culture but play a major role in viral pathogenesis (Gerrard *et al.*, 2007; Ikegami *et al.*, 2009; Müller *et al.*, 1995; Won *et al.*, 2007). *Orthobunyaviruses* lacking NSs proteins are likely nonpathogenic for humans (Mohamed *et al.*, 2009). The NSs proteins of phlebo- and orthobunyaviruses (RVFV, BUNV, LACV) efficiently inhibit type I interferon synthesis and are relevant for infection of mammals (Bird *et al.*, 2011; Blakqori *et al.*, 2007; Bouloy *et al.*, 2001; Ikegami *et al.*, 2009; Weber *et al.*, 2002). NSs and NSm proteins might thus have been acquired convergently by bunyaviruses during adaptation to vertebrate hosts. This matches our observations that GOLV could not be passaged to vertebrate cells, suggesting that the virus might depend entirely on insects rather than vertebrates for maintenance in nature (Tesh, 1988). Indeed, our finding of GOLV in two pools of male mosquitoes suggests transovarial or transvenereal transmission. This idea is supported by the existence of an NSs protein in SFTSV that is phylogenetically placed between GOLV and phleboviruses and that can infect vertebrates (Yu *et al.*, 2011).

To provide an estimate of genetic diversity within GOLV viruses, the coding regions for Gn and Gc proteins were sequenced from eight randomly chosen isolates. The isolates were clearly diversified, with a maximal distance of 4.1% at the amino acid level [Figure I.3b and c].

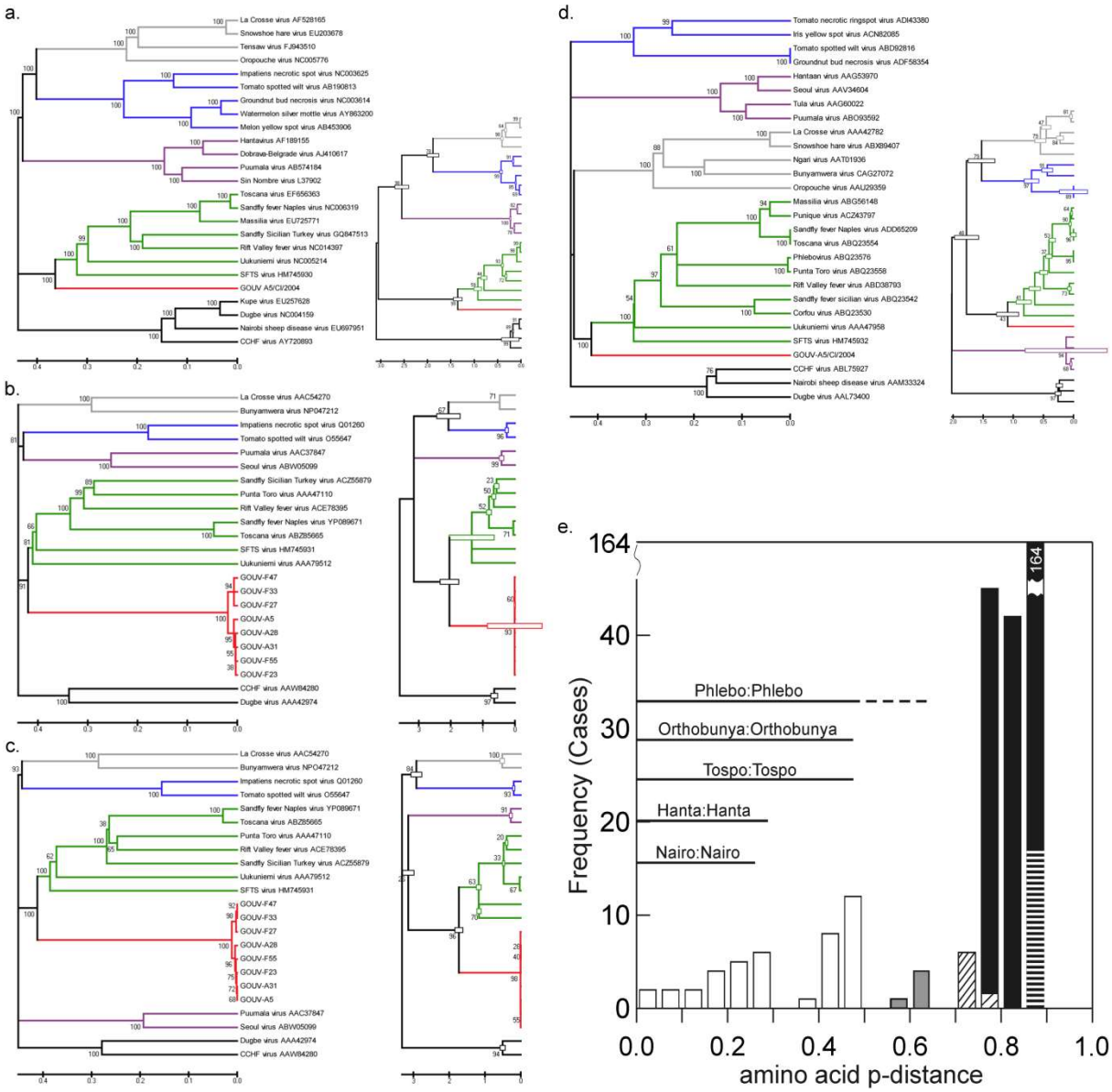

Figure I.3. Relationship of GOLV to other bunyaviruses. Phylogenetic analyses including representative members of all *Bunyaviridae* genera were performed on a gap-free amino acid alignment guided by the BLOSUM62 substitution matrix, using the neighbor-joining (NJ) algorithm with a uniform-rates substitution model and confidence testing by 1,000 bootstrap replicates in MEGA version 5.0 (Tamura *et al.*, 2011). Maximum-likelihood (ML) analyses were performed with the Dayhoff substitution model and are shown in smaller scale on the right. Phylogenies were investigated for the RdRp (a), Gn (b), Gc (c), and N (d) protein genes. Bars indicate evolutionary substitutions per position in the alignment. (e) Distribution of pairwise amino acid sequence distances between putative RdRp proteins in the family *Bunyaviridae*. A distance matrix of pairwise identity values was calculated with MEGA 5.0 (Tamura *et al.*, 2011) for 28 L-segment sequences. For each range of identity values (*x* axis), the incidence in the matrix is plotted on the *y* axis. White bars indicate pairwise distances between viruses of same genera (intragenus), and black bars indicate pairwise distances between viruses of different genera (intergenus). Pairwise distances between Uukuniemi virus and main-group phleboviruses (sandfly fever group) are shaded in gray. Pairwise distances between Gouléako virus and phleboviruses are hatched, and ranges of pairwise distances between GOLV and orthobunya-, hanta-, nairo-, and tospoviruses are marked by horizontal bars. Horizontal lines indicate ranges of pairwise sequence distances within each of the five established genera of the family *Bunyaviridae*. CCHF virus, Crimean-Congo hemorrhagic fever virus.

Phylogenetic analysis yielded five major clades reflecting the established *Bunyaviridae* genera and GOLV as an additional clade [Figure I.3a to d]. GOLV was placed in a basal phylogenetic relationship to the *Phlebovirus* genus and was more distant from the SFG than

UUKV and SFTSV were, which already constitute outliers within the genus *Phlebovirus* (Bishop *et al.*, 1980).

To characterize the amino acid distance pattern within the family *Bunyaviridae*, a distance matrix using the complete RdRP ORFs was calculated (it should be noted that this analysis excluded SFTSV, due to its unclassified status). Viruses within genera were found to be up to 47% distant from each other, except for UUKV, which showed between 57 and 61% distances from the SFG of phleboviruses [Figure I.3e]. Intergenus pairwise distances ranged between 77 and 90%. GOLV was approximately equidistant from all bunyaviruses, with distances ranging between 74 and 88%.

To examine the antigenic distinction of GOLV from the genus *Phlebovirus*, immunofluorescence assays were done on GOLV-infected cells, using antisera against a broad panel of prototypic phleboviruses, including SFV Toscana, Sicilia, and Naples strains and RVFV, as well as UUKV. No cross-reactivity was detected, while all controls showed reaction patterns as expected [Figure I.4].

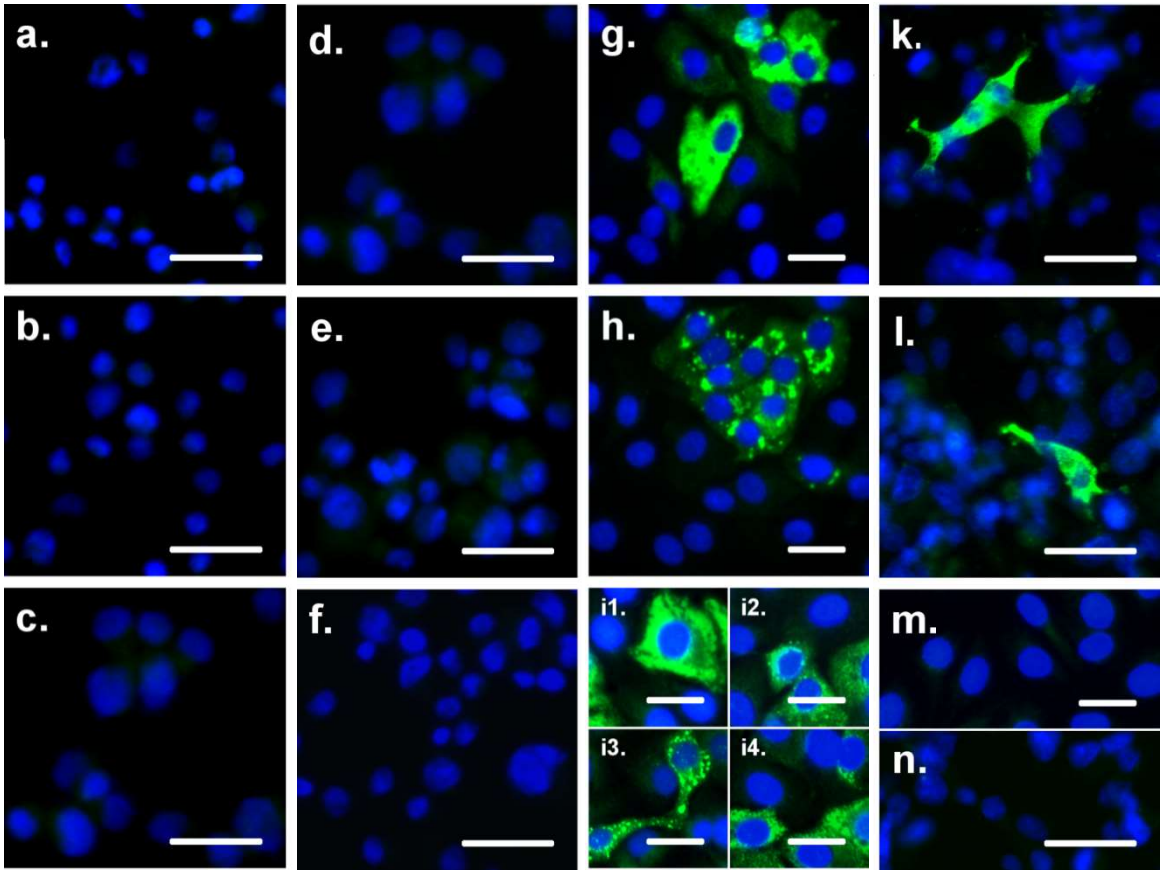

Figure I.4. Indirect immunofluorescence assay (IIFA) with GOLV and phleboviruses. GOLV-infected C6/36 cells were used to prepare slides for immunofluorescence assays. GOLV infection was confirmed by determination of infectious particles (5.0 x 10^4 TCID50/ml) and by measurement of virus RNA copies/ml (5.27 x 10^{11}/ml). GOLV-infected cells were tested with mouse anti-RVFV serum (a), mouse anti-RVFV nucleocapsid serum (b), human anti-sandfly fever virus serum (Euroimmun AG, Lübeck, Germany) (c), mouse anti-UUKV serum (9b) (d), and mouse anti-UUKV serum (8b) (e). Reactivity of all sera was confirmed on IFA slides spotted with EU14 cells infected with each respective virus. These slides were taken from the commercially available "Sandfly Fever Virus Mosaic 1" and "*Phlebovirus* Mosaic 1" detection kits (Euroimmun AG, Lübeck, Germany). These positive controls are shown as follows: mouse anti-RVFV serum (g), mouse anti-RVFV nucleocapsid serum (h), human anti-Sandfly fever Cyprus virus (SFCV) serum (i1), human anti-Sandfly fever Naples

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virus (SFNV) serum (i2), human anti-Toscana virus (TOSV) serum (i3), and human anti-SFSV serum (i4). Additional control experiments were done by incubation of 2 different mouse anti-UUKV sera (designated 8b and 9b) on IFA slides spotted with UUKV-infected BHK-21 cells, as shown in panels k and l. Experiments with negative controls were performed using uninfected C6/36 cells incubated with human anti-sandfly fever virus serum (f), uninfected EU14 cells incubated with human antisandfly fever virus serum (m), and uninfected BHK-21 cells incubated with mouse anti-UUKV serum (9b) (n). IFA detection of human and murine sera, respectively, was performed with an anti-human IgG conjugate (Euroimmun AG, Lübeck, Germany) and with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse serum (Sifin, Berlin, Germany). Cells were stained with DAPI (4',6-diamidino-2-phenylindole). Bars, 20 µm (C6/36 cells) and 50 µm (EU14 and BHK-21 cells). All photographs were taken at equivalent exposure settings.

In summary, we have identified a prototypic mosquito-associated bunyavirus that differs from the established bunyavirus genera in all taxonomically relevant genetic features and that is antigenically distinct. We assume that GOLV defines a novel genus within the family *Bunyaviridae*.

Chapter II

Discovery of a Unique Novel Clade of Mosquito-Associated Bunyaviruses

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II.1 INTRODUCTION

The family *Bunyaviridae* is among the largest and most diversified families of RNA viruses, comprising more than 350 serologically distinct viruses (Plyusnin *et al.*, 2012). Ninety-six viruses have been formally classified as distinct species by the ICTV, and full genome sequences are yet to be determined for the majority of isolates (Plyusnin *et al.*, 2012). The family comprises five genera whose members can cause pathogenic infections in vertebrates (genera *Hantavirus*, *Nairovirus*, *Orthobunyavirus*, and *Phlebovirus*) and plants (genus *Tospovirus*). Several bunyaviruses are considered emerging and reemerging pathogens due to their recent invasion of new habitats and increasing incidence in humans or livestock, such as CCHFV, RVFV, Sin Nombre virus (SNV), SFTSV, and SBV (Beer *et al.*, 2013; Bird & Nichol, 2012; Ergonul, 2012; Soldan & González-Scarano, 2005; Watson *et al.*, 2014; Yu *et al.*, 2011). Orthobunyaviruses, phleboviruses, and nairoviruses are transmitted to their vertebrate hosts by mosquitoes, midges, phlebotomine sandflies, and ticks. The genus *Hantavirus* is unique in that its members have no arthropod vectors but are transmitted by aerosolized rodent excreta (Tsai, 1987).

Bunyaviruses share general features such as their overall virion morphology or their ability to replicate in the cytoplasm and bud into the Golgi cisternae (Kuismanen *et al.*, 1982, 1984; Murphy *et al.*, 1973; Novoa *et al.*, 2005; Salanueva *et al.*, 2003). Criteria to classify bunyaviruses into genera can be derived from more specific properties such as genome organization, coding strategies, as well as phylogenetic relationships (Plyusnin *et al.*, 2012). Members of each genus are further subdivided by serology into serogroups and antigenic complexes. Phylogenetic relationships are generally in good agreement with antigenic classification, justifying the use of sequence information as the major criterion for classification of bunyavirus genera (Plyusnin *et al.*, 2012). Branching inconsistencies within genera have become evident by comparing phylogenetic relationships based on different genes, revealing a potential for bunyaviruses to undergo intrageneric genome segment reassortment (Briese *et al.*, 2007; Yanase *et al.*, 2010, 2012).

The enveloped, spherical bunyavirus virions are ca. 100 nm in diameter and contain segmented, single-stranded, negative-sense RNA genomes implementing negative-sense or ambisense coding strategies (Schmaljohn & Nichol, 2007). The S segment encodes the N protein. The M segment codes two glycoproteins (Gn and Gc), and the L segment encodes the RdRp. The S andMsegments of the genera *Orthobunyavirus*, *Phlebovirus*, and *Tospovirus* encode two additional nonstructural proteins, NSs and NSm, respectively. Orthobunyaviruses encode their N and NSs proteins in overlapping ORFs translated from one same mRNA that is complementary to the corresponding virion RNA segment (Eshita *et al.*, 1985). Phleboviruses and tospoviruses use an ambisense coding strategy and translate their NSs from a subgenomic mRNA (sg mRNA), which has the same polarity as the vRNA (Ikegami *et al.*, 2005). Recently, it was shown that some hantaviruses also code for an NSs protein in an ORF overlapping the N ORF, with expression enabled by ribosomal leaky scanning (Jääskeläinen *et al.*, 2007; Vera-Otarola *et al.*, 2012). Accessory proteins are not consistently represented throughout genera, as M segments of tick-transmitted phleboviruses do not

encode NSm proteins (Palacios *et al.*, 2013; Rönnholm & Pettersson, 1987; Yu *et al.*, 2011), and viruses in the Anopheles A, Anopheles B, and Tete virus serogroups within the genus *Orthobunyavirus* do not encode NSs proteins (Mohamed *et al.*, 2009). Bunyavirus NSs proteins either inhibit the cellular interferon response in their vertebrate hosts or suppress the RNAi mechanism in their plant hosts (Bouloy *et al.*, 2001; Bridgen *et al.*, 2001; Takeda *et al.*, 2002). *Nairoviruses* are special regarding their strategy to counteract the antiviral host response, as they code for an OTU domain within their L protein that has been suggested to suppress the host cell inflammatory and antiviral response and thus plays a role as a pathogenicity factor (Frias-Staheli *et al.*, 2007; Honig *et al.*, 2004; Kinsella *et al.*, 2004).

Bunyaviruses are distributed worldwide but appear to have higher diversity and prevalence in tropical and subtropical regions (Schmaljohn & Nichol, 2007). Investigations of bunyaviruses in such regions can yield novel insights into phylogeny and diversity. For instance, Gouléako virus (GOLV) (previously GOUV; the abbreviation was changed as GOLV was already used for Gou virus, a hantavirus isolated from Rattus rattus in China) (Plyusnina *et al.*, 2009), recently discovered in mosquitoes, is almost equidistant phylogenetically to the five established genera but closest to the genus *Phlebovirus* (Marklewitz *et al.*, 2011). Gouléako virus appears to be restricted to arthropod hosts, while all other known phleboviruses can also infect specific vertebrate hosts, suggesting that Gouléako virus represents a new taxonomic entity, potentially a new genus (Marklewitz *et al.*, 2011).

During a pilot study on mosquito-associated viruses in Côte d'Ivoire, a short RT-PCR fragment of a putative RdRp gene with a distant relationship to bunyaviruses was encountered (Junglen *et al.*, 2009a). The virus was tentatively named Herbert virus (HEBV) (strain F23/CI/2004). Here we provide a full characterization of the virus isolated in cell culture as well as related viruses isolated from mosquitoes in Côte d'Ivoire, Ghana, and Uganda.

II.2 MATERIALS AND METHODS

II.2.1 Mosquito Collection and Species Identification

Mosquitoes were trapped from February to June 2004 in Taï National Park, Côte d'Ivoire (Junglen *et al.*, 2009a) and from February to June 2008 in Kibale National Park, Uganda. Habitat types included primary and secondary tropical forests, agricultural plantations, villages, and research camps within primary rainforests. Furthermore, mosquitoes were collected at the botanical garden and at the residential area at the Kwame Nkrumah University of Science and Technology (KNUST) in Kumasi, Ghana. Mosquitoes were trapped with CDC miniature light and gravid traps (John W. Hock Company, USA) and with BG sentinel traps (Biogents, Regensburg, Germany). Traps were baited with octenol, worn socks, Limburger cheese, or simple syrup (1 liter of water mixed with 100 g sugar). Species were identified by morphological criteria (Edwards FW, 1941; Gaffigan *et al.*, 2013; Gillies & de Meillon, 1968; Jupp, 1996). Virus isolation, purification, and growth. Virus isolation from

mosquitoes collected in Côte d'Ivoire was done with C6/36 (derived from *Aedes albopictus* larvae) (Igarashi, 1978) and VeroE6/7 (*Cercopithecus aethiops* kidney) cells as described previously (Junglen *et al.*, 2009a, b). Female mosquitoes from Uganda and Ghana were homogenized individually in 500 µl of L-15 medium without additives by using 3 to 5 ceramic beads and a TissueLyser instrument (Qiagen, Hilden, Germany). Trapped male mosquitoes were pooled (1 to 20 specimens) according to trapping location and genus and homogenized in 1 ml of L-15 medium. Suspensions were cleared from debris by centrifugation at 2,500 rpm for 10 min at 4°C. Pools of female mosquitoes were generated by using 100 µl of supernatant of 10 homogenized mosquito suspensions and used for virus isolation as described previously (Junglen *et al.*, 2009b). Virus stocks of the fourth passage of HEBV (isolate C60/CI/2004) and KIBV (isolate P07/UG/2008) were generated. Virus titers were determined by $TCID_{50}$ titration, and virus-positive wells were identified by real-time PCR. For virus growth kinetics, C6/36 and U4.4 (derived from *Aedes albopictus* larvae (Singh KRP, 1967)) cells were infected at MOI of 0.1 and 0.01 in duplicate, respectively, as described previously (Zirkel *et al.*, 2011). Aliquots of infectious cell culture supernatants were harvested every 24 h for periods of 5 days, and viral genome copy numbers were quantified by real-time RT-PCR (HEBV-F [5'-AGAATGCTTTGTCAGTGG], HEBV-R [5'-AGCAGCAACTTATAAAACAAATC], HEBV-TM [5'-6-carboxyfluorescein {FAM}-TTCTCCGCTAATAAAA–MGB], KIBV-F [5'-TAATTTGAATG GTGAGCCTTTTTCT], KIBV-R [5'-GCTGTCTGAATACCGGATAAT CTTG], and KIBV-TM [5'-FAM-ATTCCCTGTCATTGGAGCTTGCTC TTTCTT-TQ2]).

II.2.2 Infection of Vertebrate Cells

Green monkey kidney cells (VeroE6/7), baby hamster kidney cells (BHK-J), mouse embryo fibroblasts (MEFs) from BALB/c $MDA5^{-/-}$ mice, MEFs from BALB/c $RIG\text{-}I^{-/-}$ mice, mouse fibroblasts (L929), and porcine stable equine kidney (PSEK) cells were infected with HEBV (fourth passage of isolate F23/CI/2004) at MOIs of 10, 1, 0.5, and 0.1 and incubated at 33°C and 37°C. Cell culture supernatants were passaged in fresh cells every 7 days in 1/10 dilutions for five consecutive passages. Supernatants from identical cell culture types infected at different MOIs were pooled, and all passages were subjected to screening by real-time RT-PCR.

II.2.3 RT-PCR Screening

RNA was extracted from homogenized female and male mosquito pools or from individually homogenized female mosquitoes using 140 µl of the supernatant and a viral RNA kit (Qiagen, Hilden, Germany), and cDNA was synthesized by using SuperScriptII according to the manufacturer's instructions (Invitrogen, Karlsruhe, Germany). Pools were screened by real-time RT-PCR or by nested RT-PCR using primer pair HEBV-F1 (5'-ATGCTGAYATGTCIAAGTGGTSTGC) and HEBV-R1 (5'-TGATTGTCATCGSTRTGIACYA) for the first

round and primer pair HEBV-F2 (5'-ATGCTGAYATGTCIAAGTGGTSTGC) and HEBV-R2 (5'-TCAARTTVCCTTGGAKCCART) for nested PCR.

II.2.4 Electron Microscopy

For electron microscopy (EM) analyses, viral particles were purified through a 36% sucrose cushion, and the pellet was resuspended in phosphate-buffered saline (PBS) (Junglen *et al.*, 2009b; Quan *et al.*, 2010). Viral particles were fixed with 2% paraformaldehyde and analyzed by transmission electron microscopy after staining with 1% uranyl acetate (Biel & Gelderblom, 1999; Hayat MA, 2000). For ultrathin sections, infected cells were fixed with 2.5% glutaraldehyde, enclosed in low-melting agar, embedded in resin, and evaluated by transmission EM after ultrathin sectioning (Junglen *et al.*, 2009b).

II.2.5 Genome Sequencing

Viral genome fragments from infectious cell culture supernatants of HEBV were generated by random-primed RTPCR optimized for the detection of encapsidated nucleic acids (so-called “particle-associated nucleic acid PCR” (Junglen *et al.*, 2009b; Marklewitz *et al.*, 2011)). Briefly, RNA was extracted from ultracentrifuged virus pellets by using the viralRNAkit (Qiagen, Hilden, Germany), and double-strand cDNA was synthesized with random hexamers linked to a defined primer sequence tail by using a double-strand cDNA kit (Promega, Madison, WI, USA). Amplification was performed by using oligonucleotides that bound to the sequence tail and were cloned into the pCR2.1 TOPO vector (Invitrogen, Karlsruhe, Germany). Colonies were analyzed by PCR, and inserts of ~500 nucleotides (nt) were sequenced by using dye terminator chemistry (Applied Biosystems, Darmstadt, Germany). Primer sequences were trimmed, and sequences were assembled by using Geneious 6 (Kearse *et al.*, 2012). Consensus sequences were compared at the nucleotide and translated amino acid levels to the GenBank database by applying BLASTn and BLASTx algorithms (http://www.ncbi.nlm.nih.gov/genbank). Fragment-specific primers and generic orthobunyavirus oligonucleotides were used for amplification of sequence gaps. The 3' and 5' genome termini were confirmed by RACE-PCR (Roche, Mannheim, Germany). The complete genome was resequenced for confirmation on both strands by long-range PCR and primer walking techniques. Full-genome sequencing of KIBV was performed by using fragment-specific primers and primers based on the HEBV genome. Full genome sequences of HEBV isolates F33/CI/2004, F45/CI/2005, and F53/CI/2004, as well as that of TAIV isolate F47/CI/2004, were generated by deep sequencing on the 454 Junior (Roche) and Ion Torrent (Invitrogen) platforms in Bonn, Germany. Reads were identified by reference mapping to HEBV F23/CI/2004 as well as by BLAST comparisons against a local amino acid sequence library containing translations of ORFs detected in the HEBV and KIBV genomes.

Table II.1. Mosquito species infected with HEBV, TAIV, or KIBV

Virus / strain	Mosquito species	# M	Sampling site	% Pairwise identity to HEBV F23/CI/2004
HEBV				
A11/CI/2004	*Cx. Eum. spp.*	20	Camp	94.8
A18/CI/2004	*Anopheles spp.*	1	Camp	96.3
A26/CI/2004	*Cx. nebulosus*	10	Camp	95.4
A27/CI/2004	n.d.	1	Camp	95.2
A28/CI/2004	*Cx. nebulosus*	22	Camp	95.7
A30/CI/2004	*Ur. mashonaensis*	6	Camp	95.8
A45/CI/2004	*Cx. telesilla*	11	Camp	95.8
A52/CI/2004	n.d.	8	Camp	96.3
A57/CI/2004	*Cx. spp.*	10	Camp	96.4
B40/CI/2004	n.d.	2	Pimary forest	96.1
B42/CI/2004	*Cx. spp.*	9	Pimary forest	95.9
C40/CI/2004	*Ur. mashonaensis*	20	Secondary forest	95.2
C43/CI/2004	*Cx. nebulosus*	17	Secondary forest	96.2
C45/CI/2004	*Cx. nebulosus*	16	Secondary forest	95.8
C57/CI/2004	*Cx. decens*	20	Secondary forest	95.9
C59/CI/2004	*Cx. decens*	20	Secondary forest	97.1
C60/CI/2004	*Cx. decens*	9	Secondary forest	97.1
C68/CI/2004	*Cx. spp.*	21	Secondary forest	96.2
C88/CI/2004	n.d.	20	Secondary forest	96.3
D24/CI/2004	*Cx. spp.*	23	Plantation	95.7
D28/CI/2004	*Anopheles spp.*	2	Plantation	95.4
D50/CI/2004	*Cx. nebulosus*	20	Plantation	96.5
D60/CI/2004	n.d.	15	Plantation	98.3
D61/CI/2004	n.d.	11	Plantation	94.6
D62/CI/2004	*Cx. spp.*	14	Plantation	96.2
F23/CI/2004	*Cx. nebulosus*	20	Village	100
F25/CI/2004	*Cx. nebulosus*	21	Village	95.8
F26/CI/2004	*Cx. nebulosus*	50	Village	95.1
F27/CI/2004	*Cx. nebulosus*	40	Village	96.7
F28/CI/2004	*Cx. nebulosus*	20	Village	96.1
F30/CI/2004	*Cx. nebulosus*	20	Village	96.5
F32/CI/2004	*Cx. nebulosus*	15	Village	96.2
F33/CI/2004	*Cx. nebulosus*	12	Village	96.1
F43/CI/2004	*Cx. spp.*	1	Village	96.2
F45/CI/2004	*Cx. spp.*	26	Village	95.8
F47/CI/2004	*Culicidae spp.*	10	Village	95.7
F53/CI/2004	*Cx. quinquefasciatus*	8	Village	96.1
F54/CI/2004	*Cx. antenatus*	20	Village	96.3
F55/CI/2004	*Cx. antenatus*	9	Village	96.1
M257/P13/GH/2011	*Cx. quinquefasciatus*	1	Residential area	95.4
M538/P27/GH/2011	*Cx. nebulosus*	1	Botanical garden	96.7
M540/P27/GH/2011	*Cx. nebulosus*	1	Botanical garden	95.9
M566/P29/GH/2011	*Cx. nebulosus*	1	Botanical garden	100
M569/P29/GH/2011	*Cx. nebulosus*	1	Residential area	95.9
M572/P29/GH/2011	*Cx. nebulosus*	1	Residential area	96.3
M105/P06/GH/2011	*Cx. pipiens*	1	Residential area	96.6
M120/P06/GH/2011	*Cx. pipiens*	1	Residential area	96.6
M201/P11/GH/2011	*Cx. quinquefasciatus*	1	Residential area	95.4
M206/P11/GH/2011	*Cx. quinquefasciatus*	1	Residential area	97.1
M211/P11/GH/2011	*Cx. quinquefasciatus*	1	Botanical garden	96.2

M213/P11/GH/2011	*Cx. quinquefasciatus*	1	Residential area	97
M219/P11/GH/2011	*Cx. quinquefasciatus*	1	Residential area	97.1
M858/P43/GH/2011	*Cx. nebulosus*	1	Botanical garden	94.9
TAIV				
C48/CI/2004	*Cx. nebulosus*	nd	Secondary forest	75.8
F47/CI/2004	*Culicidae spp.*	10	Village	76.1
KIBV				
M15/P05/UG/2008	*Cx. simpliforceps*	1	Forest edge	72.7
M22/P05/UG/2008	*Cx. simpliforceps*	1	Forest edge	72.4
M202/P07/UG/2008	*Culex spp.*	1	Tea plantation	72.4

*, Pool; M, mosquito; nd, not determined; CI, Côte d'Ivoire; GH, Ghana; UG, Uganda

II.2.6 Genome and Phylogenetic Analyses

Nucleotide and amino acid sequences were compared with other sequences by BLASTn and BLASTx against the GenBank database (http://www.ncbi.nlm.nih.gov/genbank), and protein motifs were identified by a Web-based comparison to the Pfam database (http://pfam.janelia.org/). Identification of cleavage sites of the signal peptide was accomplished by using signalP-NN (http://www .cbs.dtu.dk/services/SignalP/). Prediction of the hydropathy profile was performed with TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/), and N-linked glycosylation sites were identified by using the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/). For phylogenetic analyses, amino acid sequences of the N, Gn, Gc, and RdRp genes were aligned with representative sequences of other bunyaviruses in Geneious by using MAFFT (Katoh *et al.*, 2002). Phylogenetic analyses were conducted by using the ML algorithm with the BLOSUM62 substitution matrix assuming no systematic rate variation across alignment sites, with confidence testing based on 1,000 bootstrap iterations in PhyML (Guindon & Gascuel, 2003). Sequence alignments used for phylogenies, including all bunyavirus genera, were 587 aa, 140 aa, 622 aa, and 364 aa in length for the N, Gn, Gc, and RdRp proteins, respectively, from which the least conserved columns were removed before analysis. Phylogenetic analyses including HEBV, TAIV, KIBV, all available orthobunyavirus, and tospovirus sequences were based on 3,228 aa, 485 aa, 520 aa, and 331 aa for the RdRp, Gn, Gc, and N proteins, respectively.

II.2.7 mRNA Analyses

C6/36 cells infected with HEBV and KIBV were harvested at 24 h postinfection (hpi). RNA was extracted by using an RNA extraction kit (Qiagen, Hilden) and analyzed by 5' RACE (Invitrogen, Karlsruhe, Germany) or by Northern blotting as described previously (Zirkel *et al.*, 2011, 2013). Digoxigenin (DIG)-labeled probes for HEBV and KIBV were generated by using primer pairs HEBV-N-F (5'-TCATCTTATACAGGAGTTCAAAGAAGCGC) and HEBV-N-R (5'-ACATGACTAAACAAGTGTGAGCCTGG), KIBV-N-F (5'-TGGCTTTAAATGGGACCCGGC) and

KIBV-N-R (5'-GCTAAACAAGTGAGCACCTGGGG), and KIBV-X1 (5'-CAA GAAGGGCATTGATCTGGTTGTC) and KIBV-X2 (5'-GCACAGGCACACATCCCCTG).

II.2.8 Protein Analyses

Proteins were analyzed as described previously (Zirkel *et al.*, 2013). Briefly, viral particles were purified by gradient ultracentrifugation on a continuous gradient of 1 to 2 M sucrose in 0.01 M Tris-HCl–4 mM Na- EDTA at 35,000 rpm (SW40 rotor; Beckman) for 22 h at 4°C. Fractions (0.4 ml each) were tested by real-time PCR, and two fractions with the largest amounts of genome copies were concentrated through a 36% sucrose cushion at 35,000 rpm (SW40 rotor; Beckman) for 2 h at 4°C. The virus pellet was resuspended in 150 µl PBS overnight at 4°C. Proteins were lysed in 4x NuPage LDS sample buffer at 70°C for 10 min and separated by SDS-PAGE on a NuPage Novex 4 to 12% Bis-Tris gel with NuPage MES SDS running buffer (Invitrogen, Darmstadt, Germany). Bands were analyzed by limited tryptic digestion and mass spectrometry using a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer. RdRp and Gc proteins were additionally analyzed by liquid chromatography mass spectrometry (LC-MS).

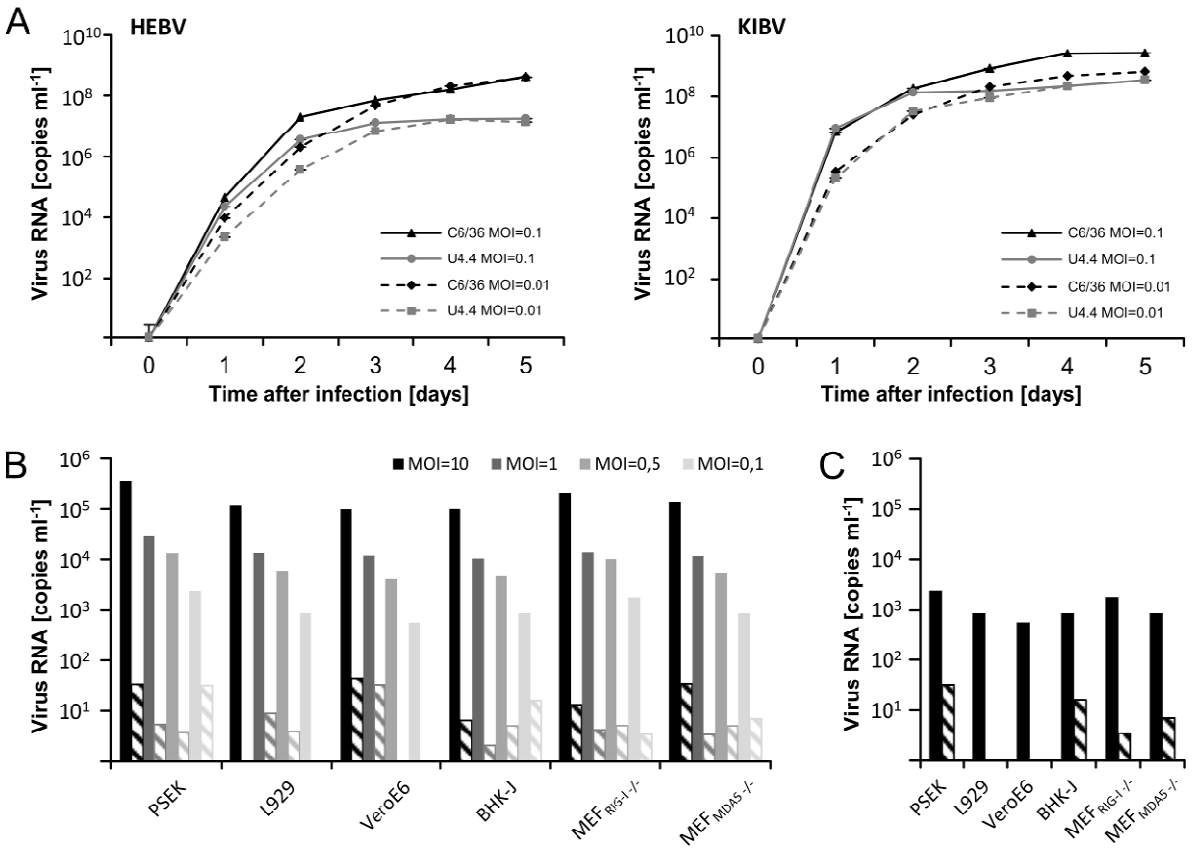

Figure II.1 Growth of HEBV and KIBV. (A) C6/36 and U4.4 cells were infected withHEBVand KIBV at MOIs of 0.1 and 0.01, respectively. The genome copy numbers per milliliter in cell culture supernatants were measured by RT-PCR for 5 days. (B) Vertebrate cells were infected with HEBV at the indicated MOIs, and five blind passages at 37°C were performed. The genome copy numbers per milliliter in cell culture supernatants were measured by RT-PCR at 7 days postinfection (solid bars) and at the fifth passage (dashed bars). (C) Cells were infected and passaged as described above for panel B but were incubated at 33°C. Supernatants of the same cell lines infected at different MOIs were pooled, and genome copy numbers were measured by RT-PCR.

II.2.9 Nucleotide Sequence Accession Numbers

The complete genome sequences of HEBV, TAIV, and KIBV were assigned GenBank accession numbers JQ659256 to JQ659258 and KF590572 to KF590586. Further sequence fragments from HEBV, TAIV, and KIBV strains of over 200 nt were assigned to GenBank accession numbers KF590587 to KF590623.

II.3 RESULTS

II.3.1 Detection of a Novel Clade of Mosquito-Associated Bunyaviruses

In order to investigate the distribution of HEBV and to detect related viruses, we tested pooled female mosquitoes collected in Taï National Park, Côte d'Ivoire (432 pools consisting of 4,839 mosquitoes); Kibale National Park, Uganda (81 pools consisting of 807 mosquitoes); and Kumasi, Ghana (62 pools consisting of 1,230 mosquitoes) by RT-PCR. HEBV was detected in 39 mosquito pools originating from Côte d'Ivoire and in 6 mosquito pools originating from Ghana, showing nucleotide distances of 94.6 to 98.3% and 94.9 to 99.2% to HEBV (strain F23/CI/2004) within their RdRp genes, respectively [Table II.1]. Individual mosquitoes from positive pools originating from Ghana were tested for infection with HEBV, resulting in a prevalence of 1.1% (14/1,230). Mosquitoes from positive pools from Côte d'Ivoire could not be tested individually, as in this case, mosquito pools had been homogenized, and no individual mosquitoes were available. Two further distinct viruses with a distant relationship at the nucleotide level to HEBV (72.6 to 72.9%) were obtained from two pools originating from Côte d'Ivoire and from two pools originating from Uganda. At the amino acid level, these viruses had distant relationships to orthobunyaviruses of the Simbu serogroup according to initial BLAST comparisons. The viruses were tentatively named Taï virus (TAIV) and Kibale virus (KIBV). Testing of individual mosquitoes from positive pools from Uganda indicated a prevalence of 0.4% (3/807). Mosquito species and sampling locations are summarized in Table II.1.

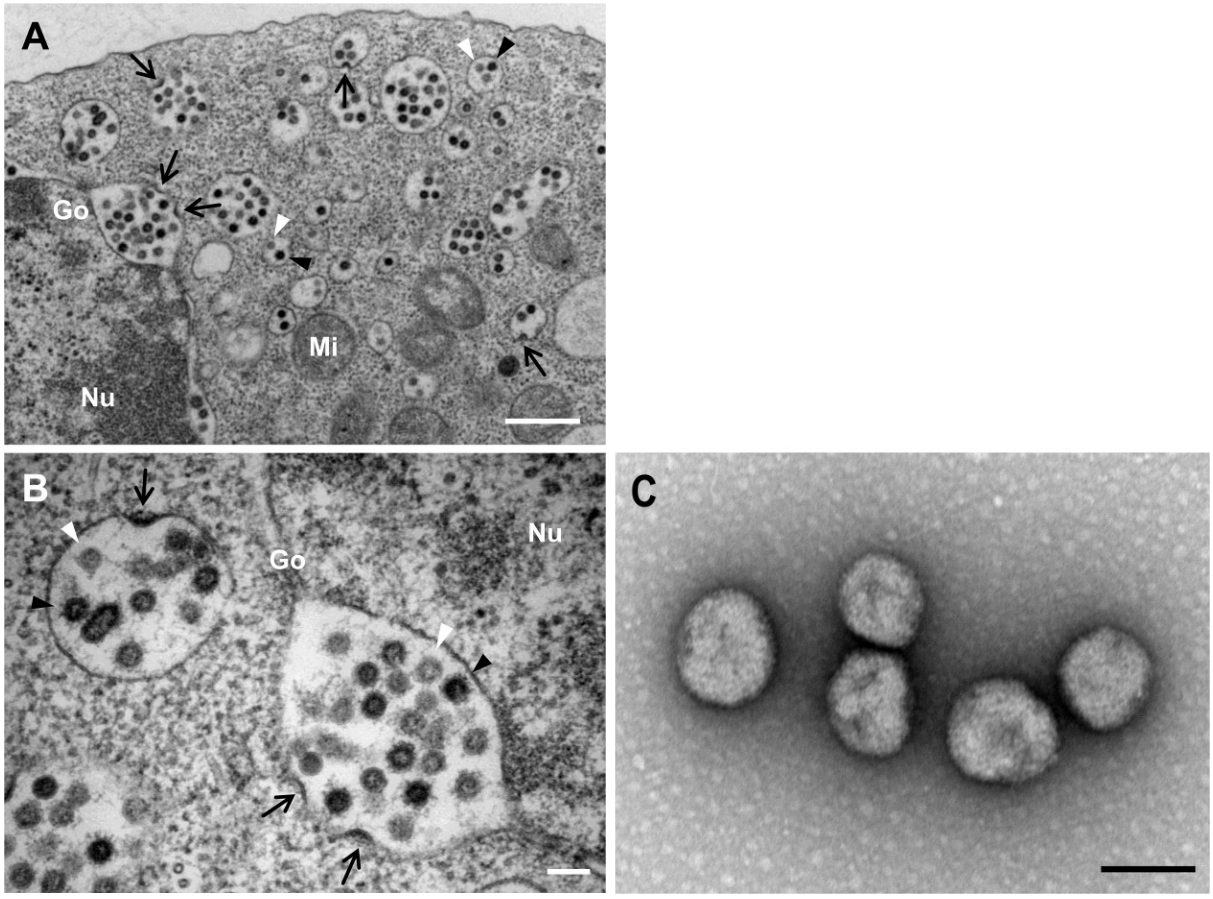

Figure II.2 Maturation and morphology of HEBV. Shown are ultrathin sections of C6/36 cells infected with HEBV (A and B) and negative-stained ultracentrifuged virions of HEBV (C). Budding arcs are indicated by black arrows, annular spherical particles are indicated by white arrowheads, and dense spherical particles are indicated by black arrowheads. Abbreviations: Nu, nucleus; Mi, mitochondria; Go, Golgi apparatus. Bars = 500nm (A) and 100nm (B and C).

II.3.2 Virus Isolation, Growth, and Morphology

HEBV was successfully isolated from 28 pools of mosquitoes in C6/36 cells. TAIV and KIBV were each isolated from two different mosquito pools, respectively. RT-PCR studies showed that both TAIV-containing cell cultures were coinfected with mesoniviruses (Zirkel *et al.*, 2013), and these could not be removed from cell cultures by repeated rounds of endpoint purification. As plaque purification was not possible due to the absence of CPE (see below), TAIV supernatants were not further purified for the purposes of this study, and growth curve studies were done only for HEBV and KIBV, for which pure supernatants were available.

HEBV (isolate C60/CI/2004) and KIBV (isolate P07/UG/2008) reached titers of 3.2 x 10^9 TCID50/ml and 3.2 x 10^7 TCID50/ml in infected C6/36 cells, respectively. Growths of HEBV and KIBV in C6/36 and U4.4 cells were compared [Figure II.1A]. For both viruses, a 10- to 100-fold-higher level of replication in C6/36 cells than in U4.4 cells was observed by 2 to 3 dpi. Notably, no CPE was observed for both viruses in U4.4 cells, and only weak changes in morphology were detected in C6/36 cells.

In order to get insight in the putative host tropism, growth of HEBV (isolate F23/CI/2004) was investigated using six different vertebrate cell lines. No CPE was observed, and no virus replication was measured by real-time RT-PCR over five blind passages in any of

these vertebrate cells [Figure II.1B and C]. Additionally, KIBV was inoculated at an MOI of 10 in Vero cells. No virus replication was detected by 7 dpi by real-time RT-PCR.

In order to assess the potential for transovarial or transvenereal transmission, we further tested 269 pools of 1,716 male mosquitoes trapped during the survey in Côte d'Ivoire, 39 pools of 386 male mosquitoes trapped in Ghana, and 11 male mosquitoes trapped in Uganda for infection with HEBV, TAIV, or KIBV. No virus was detected by RT-PCR in any of the male mosquitoes.

Virus morphology during maturation was studied in ultrathin sections of C6/36 cells infected with HEBV (isolate F23/CI/2004). Two types of spherical viral particles 50 to 60 nm in diameter, of high and low electron densities, respectively, were observed in structures resembling Golgi vesicles [Figure II.2A and B]. These were termed intracellular annular viruses (IAV) and intracellular dense viruses (IDV), in agreement with terminology used in studies on Bunyamwera virus (Salanueva *et al.*, 2003). Budding or maturation of viral particles at the Golgi membrane was observed in Golgi vesicles filled with IAV and IDV [Figure II.2A and B]. Mature spherical, enveloped virions of about 90 to 110 nm in diameter were detected in virus pellets generated by ultracentrifugation of cell culture supernatants infected with HEBV [Figure II.2C].

II.3.3 Genome Sequencing and Phylogenetic Analyses

The entire genomes of four different HEBV isolates (isolates F23/CI/2004, F33/CI/2004, F45/CI/2004, and F53/CI/2004), one TAIV isolate (isolate F47/CI/2004), and one KIBV isolate (isolate P05/UG/ 2008) were sequenced. All genomes were found to comprise three segments [Figure II.3]. Seven reverse-complementary terminal nucleotides were found to be conserved between HEBV, TAIV, and KIBV [Table II.2]. These were identical to terminal sequences in members of the genus *Orthobunyavirus*, where, however, these conserved sequences are 10 nt in length. The three genomes differed in the lengths of their UTRs of S and M segments [Figure II.3]. Pairwise nucleotide identities among all HEBV genomes ranged between 96.1 and 99.7%. Nucleotide and amino acid identities of S, M, and L segment ORFs of HEBV, TAIV, and KIBV were >61% [Table II.3].

No significant similarity was found between the S, M, and L segment ORFs and ORFs of any other viruses by using nucleotide BLAST. Low but significant levels of identity (ranging from 12 to 25%) with N protein, glycoprotein, and RdRp protein sequences of orthobunyaviruses (the most closely related virus was Oropouche virus) were identified by BLASTx using the deduced amino acid sequences of these ORFs [Table II.3].

Phylogenetic trees were inferred based on the deduced amino acid sequences of the RdRp, Gn, Gc, and N genes. Analyses of all genes, including representative sequences of established bunyavirus genera, yielded congruent topologies. HEBV, TAIV, and KIBV formed a novel independent monophyletic clade that shared the most recent common ancestor (MRCA) with the genus *Orthobunyavirus* in all genes [Figure II.4]. HEBV, TAIV, and KIBV sequences were almost equidistant to all members of the genera *Orthobunyavirus* and *Tospovirus*.

For a more detailed assessment, additional phylogenetic analyses were done including only the novel viruses as well as all orthobunyaviruses and tospoviruses, so as to avoid losses of sequence information due to indels [Figure II.4, small pictograms]. To investigate whether the novel viruses might fall into the intragenetic distance range of orthobunyaviruses or tospoviruses, pairwise identity rates for viruses the most divergent from each other of both genera were investigated. The three novel viruses showed a similar distance to each pair, indicating a similar distance to all members of both genera [Table II.3]. HEBV, TAIV, and KIBV showed mean distances of 71 to 79% to orthobunyaviruses and 81 to 86% to tospoviruses in all genes, similar to the distance between orthobunyaviruses and tospoviruses (81 to 86%).

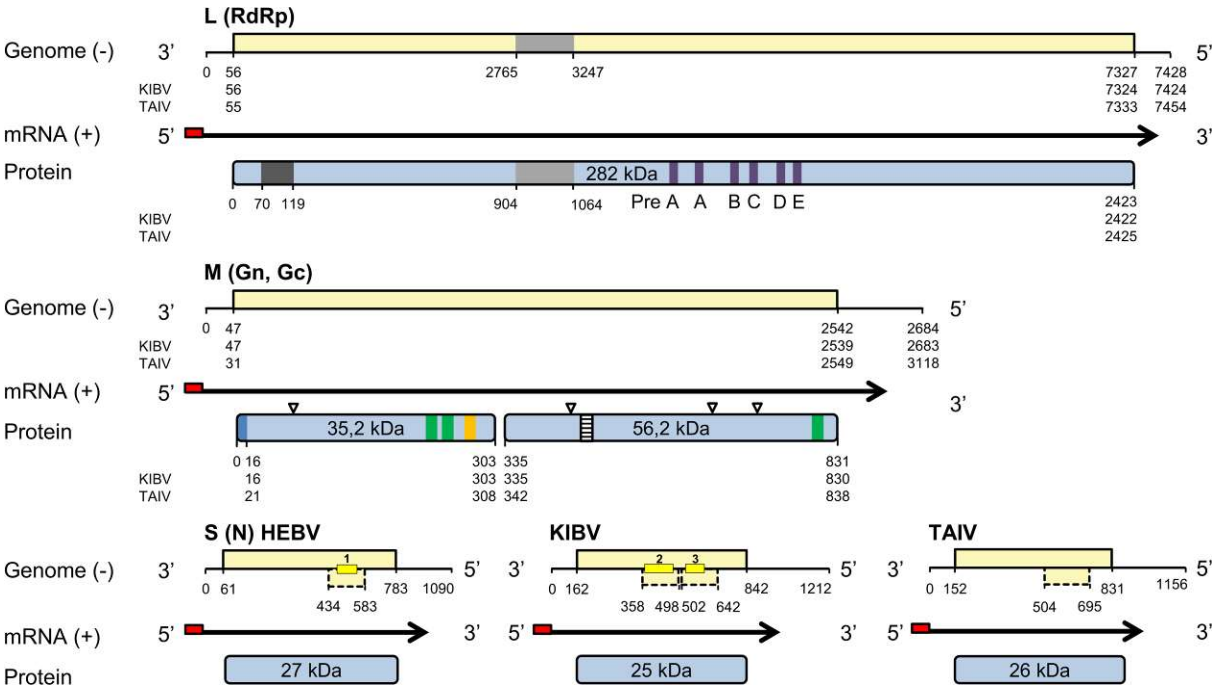

Figure II.3 Schematic view of the genome organization of HEBV, TAIV, and KIBV. Open reading frames are shown as light yellow boxes, mRNAs are indicated by black arrows, and nontemplate sequences at the 5' terminus are symbolized by red boxes. Predicted proteins are shown as light blue boxes. Northern blot probes are shown as dark yellow boxes, putative transmembrane domains (hydrophobic regions) are marked by green boxes, glycosylation sites are marked by triangles, the unique region in the RdRp gene is indicated by a light gray box, the endonuclease domain is indicated by a dark gray box, the putative signal peptide is indicated by a blue box, the Gn zinc finger motif is indicated by an orange box, and the Gc fusion peptide is indicated by a dashed box. Genome positions and predicted molecular protein masses are indicated.

II.3.4 Genome Organization of the Novel Bunyaviruses

HEBV, TAIV, and KIBV S segments comprised an ORF of 225 to 226 aa in cRNA sense that putatively encoded a 25-kDa to 27-kDa protein, presumably the N protein [Figure II.3]. No ORF was present near the N terminus of the N ORF, where an NSs protein of ca. 11 kDa is typically located in all members of the genus *Orthobunyavirus*. However, additional ORFs of 42 to 63 aa in cRNA sense were identified within the putative N ORFs of HEBV, TAIV, and KIBV [Figure II.3]. No similarities to other sequences in GenBank were detected for the smaller ORFs.

The M segments of HEBV, TAIV, and KIBV were the shortest bunyavirus M segments reported so far, about 1.2 to 1.7 kb shorter than the average size of orthobunyavirus M segments [Table II.2]. The segments contained a single ORF ranging between 830 aa and 838 aa in length that putatively encoded in cRNA sense the GPC polyprotein that is posttranslationally cleaved into the two envelope glycoproteins Gn and Gc [Figure II.3]. The GPC polyproteins in HEBV and TAIV had two possible inframe translation initiation codons ($_{47}$AUG and $_{53}$AUG, and $_{32}$AUG and $_{53}$AUG, respectively). For KIBV GPC, only one translation initiation codon at $_{47}$AUG was found. Signal peptidase cleavage sites, putative transmembrane domains (TMDs), and potential N-linked glycosylation sites of HEBV, TAIV, and KIBV are summarized in Figure II.3. Alignment of the putative GPC ORFs of HEBV, TAIV, and KIBV to the Pfam database and with orthobunyavirus glycoproteins suggested that the Gc proteins of the novel viruses were truncated by 482 aa at their N termini compared to those of orthobunyaviruses and that Gn and Gc have molecular masses of 35 kDa and 56 kDa, respectively [Figure II.3]. In contrast to orthobunyaviruses, no coding regions for putative NSm proteins were identified in all three viruses. Putative Gn zinc binding (Estrada & de Guzman, 2011) and Gc fusion peptide (Plassmeyer *et al.*, 2007) domains were identified in the predicted Gn and Gc genes of HEBV, TAIV, and KIBV, respectively [Figure II.5].

The L segments of the novel viruses were about 500 nt longer than the L segments of orthobunyaviruses due to the insertion of a unique and conserved region from amino acid positions $_{905}$LYI to $_{1064}$GLY [Figure II.3]. No significant similarity to other sequences in GenBank, including those of other bunyaviruses, was identified. A putative endonuclease domain was identified at the N termini of the L proteins in HEBV, TAIV, and KIBV (Guo *et al.*, 2012; Reguera *et al.*, 2010) [Figure II.5]. HEBV, TAIV, and KIBV were almost identical in the motifs of the third conserved region of the RdRp and exhibited the invariant residues found for bunyaviral RdRp motifs but clearly differed from members of any of the other established genera [Figure II.5].

II.3.5 Transcription Mechanism

To investigate if the novel bunyaviruses contain nontemplated sequences at their 5' ends, total RNA was analyzed from infected cells by 5'-RACE RT-PCRs with reverse primers placed on all genome segments of HEBV and KIBV. Non-virally templated sequences of 9 to 16 nt and of 10 to 22 nt were detected at the 5' ends of all HEBV and KIBV segments, respectively, indicating that viral mRNA 5' ends are formed according to the typical mechanism for bunyaviruses [Figure II.6] (Bishop *et al.*, 1983; Garcin *et al.*, 1995; Jin & Elliott, 1993; Simons & Pettersson, 1991).

Table II.2 Genome size and consensus terminal nucleotides of HEBV, TAIV, and KIBV compared to established genera of the family *Bunyaviridae*

Genus and virus	Consensus terminal nucleotides*	Genome size (nt)	Segment size (nt) (GenBank Accession no.)		
			S	M	L
Hantavirus					
Hantaan virus	3' AUCAUCAUCUG– 5' UAGUAGUAUGC–	11,845	1696 (M14626)	3616 (M14627)	6533 (X55901)
Nairovirus					
Dugbe virus	3' AGAGUUUCU– 5' UCUCAAAGA–	18,855	1712 (M25150)	4888 (M94133)	12255 (U15018)
Tospovirus					
Tomato spotted wiltvirus	3' UCUCGUUA– 5' AGAGCAAU–	16,634	2916 (D00645)	4821 (S48091)	8897 (D10066)
Phlebovirus					
Rift Valley fever virus	3' UGUGUUUC– 5' ACACAAAG–	11,979	1690 (X53771)	3885 (M11157)	6404 (X56464)
Unassigned					
Gouléako virus	3' UGUGU– 5' ACACA–	10,633	1087 (HQ541736)	3188 (HQ541737)	6358 (HQ541738)
Orthobunyavirus					
Bunyamwera virus	3' **UCAUCAC**AUG– 5' **AGUAGUG**UGC–	12,294	961 (D00353)	4458 (M11852)	6875 (X14383)
Unassigned					
Herbert virus					
S	3' **UCAUCAC**ACG– 5' **AGUAGUG**CAC–	11,202	1090	2684	7428
M	3' **UCAUCAC**ACG– 5' **AGUAGUG**CAC–				
L	3' **UCAUCAC**ACG– 5' **AGUAGUG**UGC–				
Kibale virus					
S	3' **UCAUCAC**ACG– 5' **AGUAGUG**CAC–	11,322	1212	2683	7427
M	3' **UCAUCAC**ACG– 5' **AGUAGUG**CAC–				
L	3' **UCAUCAC**ACG– 5' **AGUAGUG**CAC–				
Taï virus					
S	3' **UCAUCAC**GUG– 5' **AGUAGUG**CAC–	11,728	1,156	3,118	7,454
M	3' **UCAUCAC**GUG– 5' **AGUAGUG**CAC–				
L	3' **UCAUCAC**GUG– 5' **AGUAGUG**UGC–				

*Boldface type indicates conserved terminal nucleotides

Bunyaviruses generate three different types of RNA for replication and transcription, including negative-sense vRNA, positive-sense replicative cRNA, and mRNA species that contain 5'-methylated capped nonviral (primer) sequences and truncations at their 3' ends

compared to the vRNA and cRNA (Schmaljohn & Nichol, 2007). We did a preliminary analysis of transcription of the S segments of HEBV and KIBV by Northern blotting. Two bands each were detected for HEBV and KIBV, respectively [Figure II.7]. The larger bands likely corresponded to vRNA and cRNA occurring during viral replication, and the smaller bands likely represent viral mRNA transcription products. No shorter RNA transcripts, as would be expected in the case of transcription from hypothetical downstream promoters, were detected [refer to the placement of Northern blot probes shown in Figure II.3].

II.3.6 Major Structural Proteins

To identify the major structural proteins, HEBV particles were purified by gradient ultracentrifugation, and viral proteins were separated by SDS-PAGE before staining with Coomassie brilliant blue. Four distinct proteins, of about 280 kDa, 60 kDa, 36 kDa, and 27 kDa, were identified [Figure II.8]. MALDI-TOF mass spectroscopy confirmed two bands, corresponding to the Gn and N proteins [Figure II.8]. The RdRp and Gc proteins were identified by LC-MS because MALDI-TOF analysis yielded no conclusive results for these proteins [Figure II.8]. While migrations of the L and N proteins corresponded well with their predicted molecular masses, the bands corresponding to Gc and Gn proteins migrated at higher-molecular-mass equivalents than predicted based upon their amino acid sequences, which would be compatible with N-linked glycosylation at the sites described above [Figure II.3].

Table II.3 Nucleotide and amino acid pairwise sequence identity values for HEBV, TAIV, KIBV, and OROV as well as pairs of the most distantly related orthobunyaviruses and tospoviruses*

Gene	Virus	% nucleotide or amino acid sequence identity							
RdRp		HEBV	TAIV	KIBV	OROV	SIMV	SORV	TZSV	BeNMV
	HEBV		73.9	73.7	37.8	38.9	39.0	28.4	28.0
	TAIV	79.2		72.1	37.4	38.5	38.8	27.7	27.8
	KIBV	80.0	78.5		36.8	38.0	38.1	27.9	27.5
	OROV	24.7	24.6	24.8		60.8	56.2	27.8	27.4
	SIMV	24.7	24.6	24.6	58.2		55.7	27.3	27.3
	SORV	24.3	24.3	24.2	49.1	47.2		27.2	27.4
	TZSV	14.1	14.0	13.5	13.6	13.1	13.3		52.6
	BeNMV	13.5	14.0	13.0	14.2	12.6	12.4	41.4	
GPC		HEBV	TAIV	KIBV	OROV	AKAV	TAHV	MYSV	BeNMV
	HEBV		70.0	69.6	21.9	22.2	21.8	20.4	20.7
	TAIV	70.4		68.4	21.5	22.5	21.8	20.1	20.5
	KIBV	69.6	67.2		21.3	22.3	21.4	20.2	20.5
	OROV	12.3	12.6	12.0		43.2	42.6	26.5	26.5
	AKAV	12.0	11.5	11.7	24.7		45.0	23.6	24.3
	TAHV	10.7	11.4	11.8	31.8	29.3		23.1	22.5
	MYSV	11.6	12.5	12.4	9.8	10.9	10.3		39.3
	BeNMV	12.4	12.2	12.6	9.7	10.8	8.9	32.6	

N		HEBV	TAIV	KIBV	OROV	BMAV	BORV	TZSV	INSV
	HEBV		65.3	69.3	31.0	30.6	33.5	25.1	25.4
	TAIV	66.2		64.0	29.9	31.3	33.8	25.3	25.6
	KIBV	72.6	60.9		32.7	31.7	33.7	25.5	25.4
	OROV	19.8	20.2	20.2		38.2	37.3	24.4	27.1
	BMAV	16.5	17.4	15.9	32.1		39.7	26.9	24.3
	BORV	17.0	16.7	18.3	31.5	24.8		27.2	25.6
	TZSV	10.8	11.5	12.2	10.6	9.5	11.4		24.7
	INSV	13.2	12.0	12.7	14.6	11.7	11.5	24.9	

*Top right values for each gene indicate nucleotide sequence identity; bottom left values indicate amino acid identity. AKAV, Akabane virus; BeNMV, bean necrotic mosaic virus; BMAV, Batama virus; BORV, Boraceia virus; INSV, impatiens necrotic spot virus; MYSV, melon yellow spot virus; OROV, Oropouche virus; SIMV, Simbu virus; SORV, Sororoca virus; TAHV, Tahyna virus; TZSV, tomato zonate spot virus.

II.4 DISCUSSION

In this study, we discovered and characterized three novel bunyaviruses detected in mosquitoes from Côte d'Ivoire, Ghana, and Uganda. The data showed that HEBV, TAIV, and KIBV represent three novel bunyaviruses that do not group with any of the established bunyavirus genera. Although formal classification criteria for bunyavirus genera are not established, inferred tree topologies showed that the novel viruses form a novel phylogenetic sister group to orthobunyaviruses. Phylogenetic distances and comparisons of sequence similarity suggested these viruses to be sufficiently related to each other to classify them into one genus. In contrast, they were collectively about as distant from the established bunyavirus genera as the latter were from each other. This suggests that the novel viruses might form a separate genus. In order to generate auxiliary classification criteria, we investigated host range, viral growth and morphology, genome organization, as well as features of genome replication and gene expression.

HEBV, TAIV, and KIBV were detected in mosquitoes of three different genera (mainly *Culex nebulosus*, *Culex quinquefasciatus*, and *Culex simpliforceps*) and replicated well in RNAi-competent U4.4 cells (Attarzadeh-Yazdi *et al.*, 2009; Morazzani *et al.*, 2012) and in C6/36 cells that have impaired Dicer 2-based RNAi responses (Brackney *et al.*, 2010; Scott *et al.*, 2010; Vodovar *et al.*, 2012), indicating no growth restrictions in insect cells with an intact antiviral RNAi system. The growth phenotype in insect cells involving no or very little CPE and the inability to replicate in a large range of vertebrate cells was unexpected. Insect-restricted viruses normally cause clear CPE in insect cells. The absence of CPE in insect cells is rather typical for viruses that can additionally infect vertebrate hosts (Plyusnin *et al.*, 2012), which in turn could not be confirmed here by cell culture experiments. Notably, for the maintenance of insect-restricted viruses in nature, insect cycles involving horizontal (transvenereal) and vertical (transovarial) transmission are necessary. For instance, transovarial and transvenereal transmission to up to 30% of arthropod offspring has been described for bunyaviruses (Schopen *et al.*, 1991; Thompson & Beaty, 1977; Turell *et al.*, 1982). Some viruses can be maintained in overwintering vectors or during time periods with a low density of amplifying hosts (McGaw *et al.*, 1998; Tesh *et al.*, 1992). In contrast, in this study, we have gained no evidence for infection of any of the novel viruses in male

mosquitoes, which is a hallmark of transovarial or transvenereal transmission. Further infection studies with a larger range of vertebrate cell lines as well as ecological investigations of insects and potential amplificatory vertebrate hosts will be necessary to clarify whether the novel viruses constitute arboviruses. Critically, proof of their insect restriction would constitute a criterion to delineate the novel viruses from the genus *Orthobunyavirus*, a classical group of arboviruses employing vertebrate-based amplification.

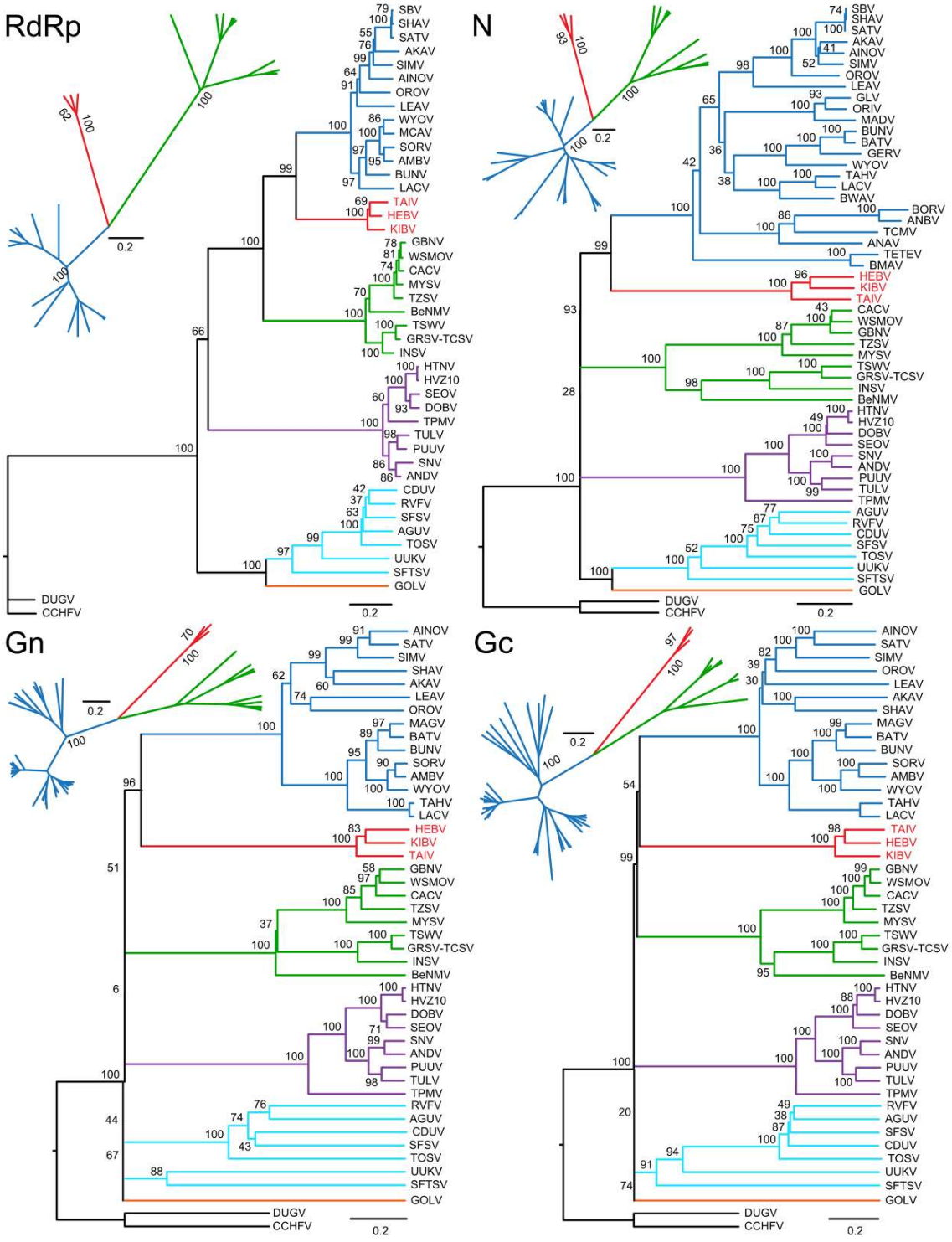

Figure II.4 (Legend continued on following page)

Phylogenetic relationships of HEBV, TAIV, and KIBV to representative members of the family *Bunyaviridae*. Phylogenies were investigated for the RdRp, Gn, Gc, and N proteins based on sizes of 364 aa, 140 aa, 622 aa, and 587 aa, respectively. Maximum likelihood (ML) analyses were performed on a gap-free alignment guided by the BLOSUM62 substitution matrix and using MAFFT (E-INS-I algorithm). Confidence testing was performed by 1,000 bootstrap replicates. Bars indicate evolutionary substitutions per position in the alignments. Smaller pictograms represent ML analyses of HEBV, TAIV, KIBV, all available orthobunyavirus, and tospovirus sequences based on sizes of 3,228 aa, 485 aa, 520 aa, and 331 aa for the RdRp, Gn, Gc, andNproteins, respectively. Abbreviations (and GenBank accession numbers for L, M, and S segments, respectively, in parentheses) are as follows: AGUV, Aguacate virus (accession numbers NC_015451, NC_015450, and NC_015452); AINOV, Aino virus (accession numbers NC_018465, NC_018459, and NC_018460); AKAV, Akabane virus (accession numbers NC_009894, NC_009895, and NC_009896); AMBV, Anhembi virus (accession numbers JN572062, JN572063, and JN572064); ANDV, Andes virus (accession numbers NC_003468, NC_003467, and NC_003466); BeNMV, bean necrotic mosaic virus (accession numbers NC_018070, NC_018072, and NC_018071); BUNV, Bunyamwera virus (accession numbers NC_001925, NC_001926, and NC_001927); CACV, Capsicum chlorosis virus (accession numbers NC_008302, NC_008303, and NC_008301); CDUV, Candiru virus (accession numbers NC_015374, NC_015373, and NC_015375); DOBV, Dobrava virus (accession numbers NC_005235, NC_005234, and NC_005233); GBNV, groundnut bud necrosis virus (accession numbers NC_003614, NC_003620, and NC_003619); GOLV, Gouléako virus (accession numbers HQ541738, HQ541737, and HQ541736); GRSV-TCSV, groundnut ringspot and tomato chlorotic spot virus reassortant (accession numbers NC_015469, NC_015468, and NC_015467); HEBV, Herbert virus (accession numbers JQ659256, JQ659257, and JQ659258); HTNV, Hantaan virus (accession numbers NC_005222, NC_005219, and NC_005218); HVZ10, Hantavirus Z10 virus (accession numbers NC_006435, NC_006437, and NC_006433); INSV, Impatiens necrotic spot virus (accession numbers NC_003625, NC_003616, and NC_003624); KIBV, Kibale virus (accession numbers KF590577, KF590576, and KF590575); LACV, La Crosse virus (accession numbers NC_004108, NC_004109, and NC_004110); LEAV, Leanyer virus (accession numbers HM627178, HM627176, and HM627177); MCAV, Macaua virus (accession numbers JN572068, JN572069, and JN572070); MYSV, melon yellow spot virus (accession numbers NC_008306, NC_008307, and NC_008300); OROV, Oropouche virus (accession numbers NC_005776, NC_005775, and NC_005777); PUUV, Puumala virus (accession numbers NC_005225, NC_005223, and NC_005224); RVFV, Rift Valley fever virus (accession numbers NC_014397, NC_014396, and NC_014395); SATV, Sathuperi virus (accession numbers NC_018461, NC_018466, and NC_018462); SBV, Schmallenberg virus (accession numbers JX853179, JX853180, and JX853181); SEOV, Seoul virus (accession numbers NC_005238, NC_005237, and NC_005236); SFSV, sandfly fever Sicilian virus (accession numbers NC_015412, NC_015411, and NC_015413); SFTSV, severe fever with thrombocytopenia syndrome virus (accession numbers NC_018136, NC_018138, and NC_018137); SHAV, Shamonda virus (accession numbers NC_018463, NC_018467, and NC_018464); SIMV, Simbuvirus (accession numbers NC_018476, NC_018478, and NC_018477); SNV, Sin Nombre virus (accession numbers NC_005217, NC_005215, and NC_005216); SORV, Sororoca virus (accession numbers JN572071, JN572072, and JN572073); TAIV, Taï virus (accession numbers KF590574, KF590573, and KF590572); TOSV, Toscana virus (accession numbers X68414, X89628, and X53794); TPMV, Thottapalayam virus (accession numbers NC_010707, NC_010708, and NC_010704); TSWV, tomato spotted wilt virus (accession numbers NC_002052, NC_002050, and NC_002051); TULV, Tula virus (accession numbers NC_005226, NC_005228, and NC_005227); TZSV, tomato zonate spot virus (accession numbers NC_010491, NC_010490, and NC_010489); UUKV, Uukuniemi virus (accession numbers NC_005214, NC_005220, and NC_005221); WSMOV, watermelon silver mottle virus (accession numbers NC_003832, NC_003841, and NC_003843); WYOV, Wyeomyia virus (accession numbers JN572080, JN572081, and JN572082).

Species within the genus *Orthobunyavirus* are classically defined by serological criteria (Plyusnin *et al.*, 2012). The genetic distance between established orthobunyavirus serogroups ranges between 27 and 53% based on glycoprotein and nucleocapsid protein amino acids. Serogroups will not serologically cross-react with each other (de Brito Magalhães *et al.*, 2011; Calisher, 1996; Saeed *et al.*, 2001; Savji *et al.*, 2011). Because the amino acid distance between the novel viruses and any orthobunyavirus ranged from 88 to 89%, and similar distances existed between orthobunyaviruses and tospoviruses, we could not expect the new viruses to yield any meaningful crossreactivities using any animal serum directed against orthobunyaviruses or tospoviruses. Serological cross-comparisons were therefore not attempted.

Various pathogenicity- and tropism-related functions of orthobunyavirus and phlebovirus NSs proteins have been found in mammalian cells, including the suppression of host protein synthesis (Billecocq *et al.*, 2004; Bridgen *et al.*, 2001; Hart *et al.*, 2009; Le May *et al.*, 2004), the inhibition of the host cell antiviral interferon response (Billecocq *et al.*, 2004; Bouloy *et al.*, 2001; Bridgen *et al.*, 2001; van Knippenberg *et al.*, 2010; Kohl *et al.*, 2003; Le May *et al.*, 2008; Streitenfeld *et al.*, 2003), as well as the inhibition of RNA polymerase II-mediated transcription (Ikegami *et al.*, 2009; Le May *et al.*, 2004; Thomas *et al.*, 2004). The inability of the novel bunyaviruses to replicate in vertebrate cells might be due to the putative absence of an NSs protein. Putative NSs proteins similar in sequence or position to those in orthobunyaviruses, tospoviruses, and phleboviruses were not identified in HEBV,

TAIV, and KIBV. The smaller ORFs located in the C-terminal half of the N ORF of the novel bunyaviruses may encode proteins of only 5 to 7 kDa, which are significantly smaller than NSs proteins of other bunyaviruses. Moreover, no mRNAs corresponding in size to the smaller ORFs were detected by Northern blotting.

Viruses of the Anopheles A, Anopheles B, and Tete serogroups were able to replicate in newborn mice and Vero cells, although these viruses were shown not to encode NSs proteins and were not able to counteract the antiviral interferon response (Mohamed *et al.*, 2009). Another group of viruses within the genus *Orthobunyavirus*, the Wyeomyia group viruses, have truncated NSs sequences that may not code for functional proteins (Chowdhary *et al.*, 2012). However, antibodies were detected in humans, and the viruses are associated with febrile illness (Aitken *et al.*, 1968; Sirhongse & Johnson, 1965; de Souza Lopes *et al.*, 1975). Determination of whether the inability of HEBV, TAIV, and KIBV to replicate in vertebrate cells is due to the absence of an NSs protein or is encoded within another genome region needs further in-depth studies.

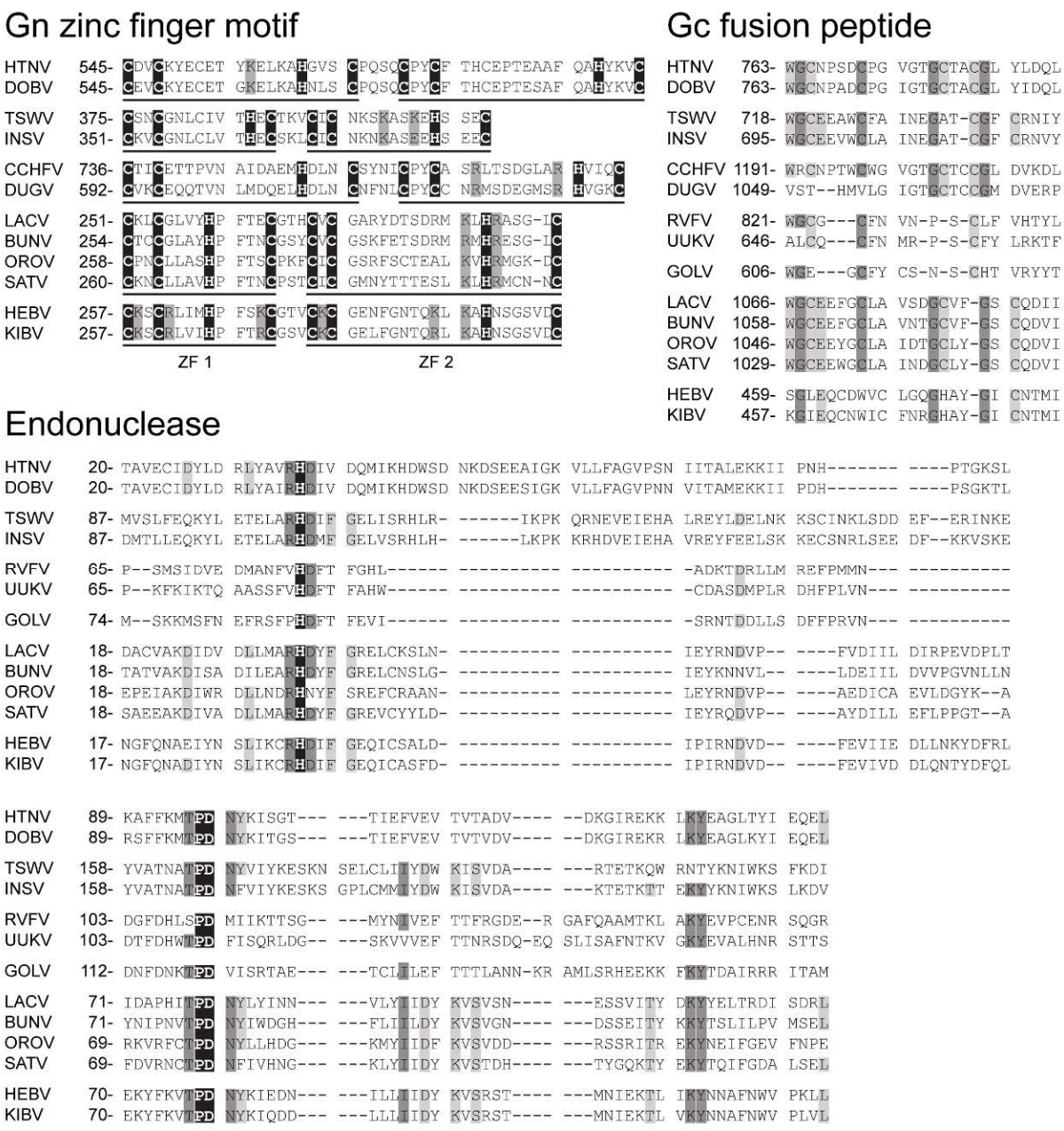

Figure II.5 (continued)

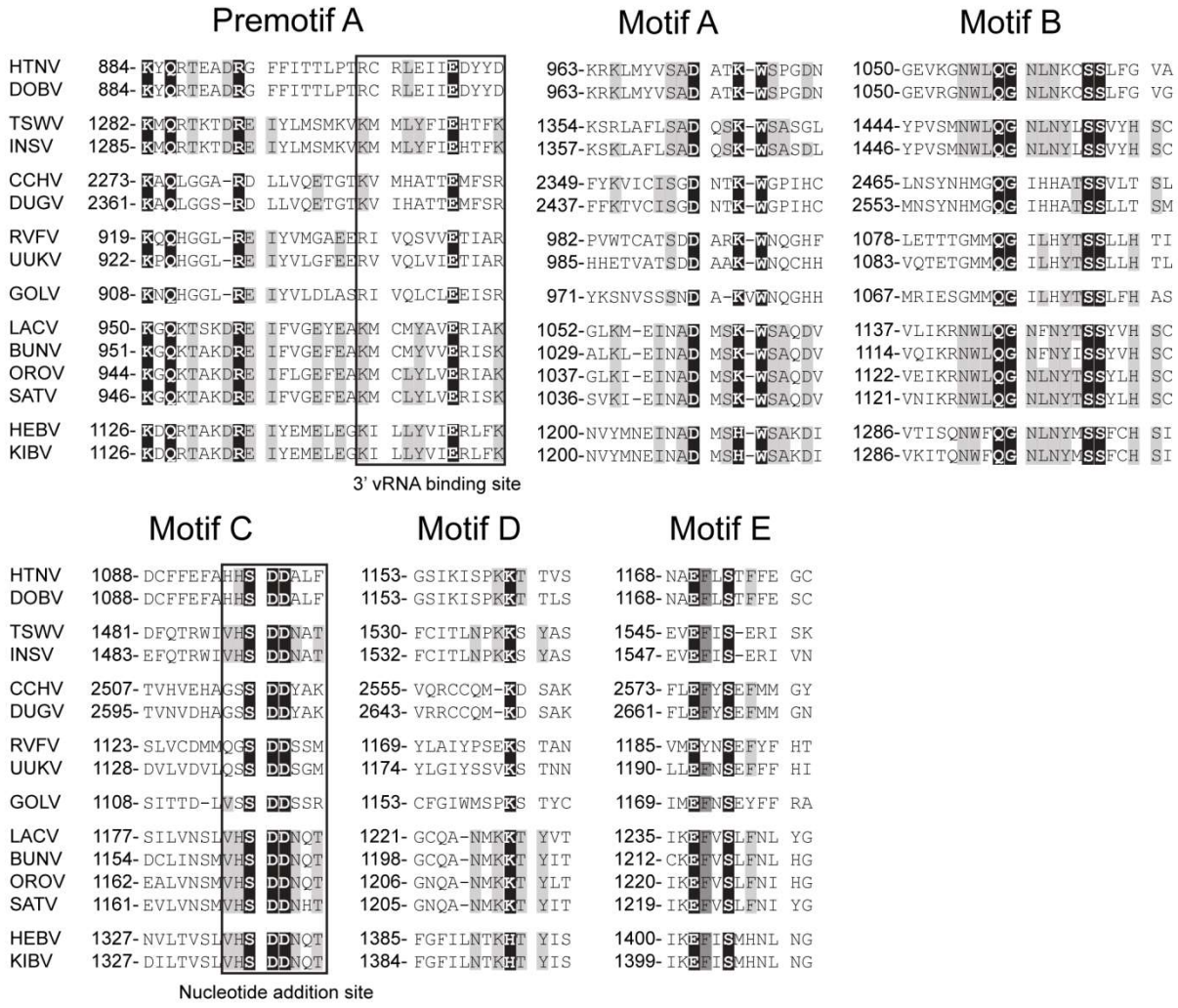

Figure II.5 Multiple-sequence alignments of conserved domains of HEBV, TAIV, KIBV, and other bunyaviruses. Alignments were performed by using the E-INS-I algorithm in MAFFT and manually edited. Numbers represent genome positions. Amino acids with 100% identity are highlighted in black, those with 75% identity are highlighted in dark gray, and those with 50% identity are highlighted in light gray. Gn zinc finger motifs are highlighted in black, and conserved basic residues are highlighted in dark gray.

The only other known nonstructural protein in bunyaviruses, the NSm protein, which was shown to play a role in the pathogenesis of Rift Valley fever virus (Bird *et al.*, 2008), was also not present in the three novel viruses. The NSm protein is encoded within orthobunyaviruses between the Gn and Gc proteins. The three proteins are expressed as a polyprotein from the M segment ORF and posttranslationally cleaved. So far, no orthobunyavirus (or tospovirus) without an NSm protein has been reported, providing an additional indication of the uniqueness of the novel viruses as a separate taxonomic entity.

There is little information on the role of NSs and NSm proteins in mosquitoes. It has been shown that the BUNV NSs protein is essential for replication in U4.4 and *Aedes aegypti* (Ae) cells and is required for replication and spread in *Aedes aegypti* mosquitoes (Szemiel *et al.*, 2012). In contrast, no specific function of the La Crosse virus NSs protein and of the Rift Valley fever virus NSs protein was found in mosquito cells and mosquitoes, respectively (Blakqori *et al.*, 2007; Crabtree *et al.*, 2012; Moutailler *et al.*, 2010). However, the NSm protein seems to be essential for replication of Rift Valley fever virus in mosquitoes

(Crabtree *et al.*, 2012). Rift Valley fever virus NSm was also found to inhibit apoptosis in mammalian cells (Won *et al.*, 2007). In contrast, viruses of the California serogroup (genus *Orthobunyavirus*) seem to induce apoptosis triggered by the NSs protein (Colón-Ramos *et al.*, 2003), a function homologous to Reaper, a *Drosophila melanogaster* protein that induces apoptosis (Goyal *et al.*, 2000; Holley *et al.*, 2002). Interestingly, sequence similarities to the Trp/GH3 motif of Reaper and the corresponding Reaper-like regions in the NSs of California serogroup viruses were identified in the L proteins of HEBV, TAIV, and KIBV ($_{283}$WRILESKLLET$_{293}$, $_{283}$WKDLETKLTST$_{293}$, and $_{283}$WKMLEEKLEK$_{293}$, respectively [conserved sequences among Reaper and HEBV, TAIV, and KIBV are underlined]). The Trp/GH3 motif is conserved among Reaper and two other Drosophila proteins, Grim and Sickle, which have crucial functions in programmed cell death (Christich *et al.*, 2002; Clavería *et al.*, 2002; Wing *et al.*, 2002). Whether this Trp/GH3-like motif in HEBV, TAIV, and KIBV may have homologous functions needs to be studied.

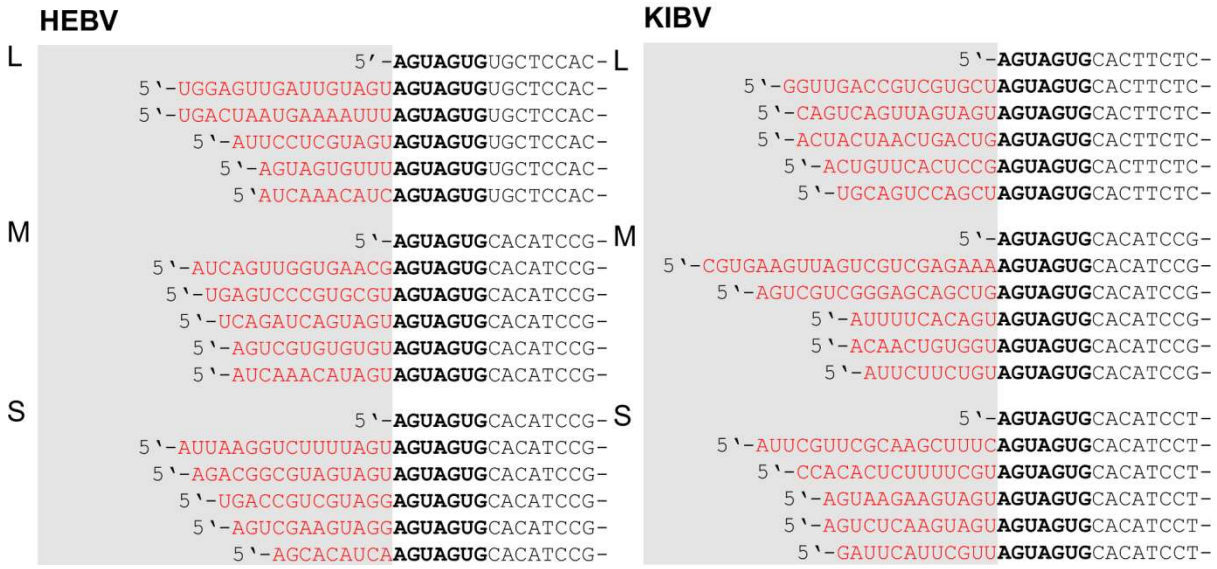

Figure II.6 Nontemplated sequences of mRNAs of HEBV and KIBV. Shown are 5' genome termini of L, M, and S segment mRNAs of HEBV and KIBV. C6/36 cells were infected with HEBV and KIBV, and total RNA was extracted at 1 dpi. Genome termini were amplified by 5' RACE-PCR, PCR products were cloned, and five random clones were analyzed. Nontemplate sequences (putative transcription primers obtained from host cell mRNAs) are marked by gray boxes. Conserved genome termini of HEBV and KIBV are shown in boldface type.

The absence of any NS protein ORFs conserved across the clade comprising tospoviruses, orthobunyaviruses, and the novel viruses suggests that the most recentcommonancestor of all of these viruses would not have encoded any of these genes. Rather, the different coding strategies for NS proteins suggest independent acquisitions during the formation of generic viral lineages. In particular, NSs and NSm proteins might have been acquired during the evolution of orthobunyaviruses in the course of acquiring replicative capability in vertebrate hosts.

A unique insertion of about 500 nt was identified in the RdRp genes of HEBV, TAIV, and KIBV. This additional region, not found in any other bunyaviruses, might represent a putative accessory protein domain. The presence of an accessory domain in the L protein is not unprecedented. For example, the CCHFV L protein contains an OTU-like cysteine

protease that has been suggested to suppress the host cell inflammatory and antiviral response (Frias-Staheli *et al.*, 2007). The L proteins of orthobunyaviruses, tospoviruses, hantaviruses, and nairoviruses contain an N-terminal endonuclease domain (Guo *et al.*, 2012; Heinemann *et al.*, 2013; Reguera *et al.*, 2010). However, no sequence similarities of the unique region in HEBV, TAIV, and KIBV to any other viral proteins were found. We further specifically searched for GW/WG motifs found to be conserved within viral RNA silencing suppressor proteins encoded by many insect-restricted viruses (Bivalkar-Mehla *et al.*, 2011). No such motifs were detected in all translated HEBV, TAIV, and KIBV ORFs. Determination of whether HEBV, TAIV, and KIBV express any accessory proteins at all will therefore require further experimental studies.

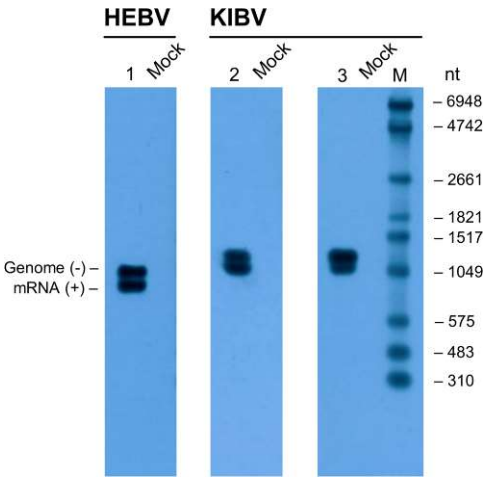

Figure II.7 S segment replication and transcription products analyzed by Northern blotting. ViralRNAwas isolated from HEBV- and KIBV-infected C6/36 cells at 2 dpi. RNA from noninfected C6/36 cells was used as a control. A DIG-labeled RNA was used as a size marker (M), with sizes given in nucleotides at the right. Positions of DIG-PCR probes are shown in Figure II.3.

While the ORFs were well conserved among HEBV, TAIV, and KIBV, the high level of variability of the UTRs and the extended length of up to 569 nt in the TAIV M segment 5' UTR were surprising. The UTRs have many different functions and play a role during replication, transcription, encapsidation, and packaging of the viral genome (Barr & Wertz, 2005; Kohl *et al.*, 2004, 2006; Osborne & Elliott, 2000). 3' and 5' UTR lengths of the three genome segments are generally well conserved among different orthobunyaviruses, with M and L segment 3' and 5' UTRs of about 50 to 100 nt and S segment 3' and 5' UTRs of about 80 to 200 nt. It will be interesting to study the functions of these highly different UTRs. Interestingly, the terminal nucleotides of the UTRs are strictly conserved among bunyaviruses of the same genus, serving as a criterion for genus classification (Plyusnin *et al.*, 2012). HEBV, TAIV, and KIBV contained unique terminal nucleotides that were truncated compared to orthobunyaviruses, precluding their grouping into the genus *Orthobunyavirus* and providing further support that the viruses constitute a separate taxonomic entity.

Segmented negative-strand RNA viruses of the families *Orthomyxoviridae*, *Bunyaviridae*, and *Arenaviridae* use capped RNA primers that are cleaved from the 5' termini of host cell mRNAs in order to initiate their transcription (Bishop *et al.*, 1983; Caton & Robertson, 1980; Garcin *et al.*, 1995; Jin & Elliott, 1993; Raju *et al.*, 1990; Simons & Pettersson, 1991). The lengths of reported capped primers vary from 10 to 20 nt (Bishop *et al.*, 1983; Caton & Robertson, 1980; Garcin *et al.*, 1995; Jin & Elliott, 1993; Raju *et al.*, 1990;

Simons & Pettersson, 1991). We found nontemplated sequences of 9 to 16 nt and 10 to 22 nt at the 5' termini of HEBV and KIBV mRNAs, respectively. Primer sequences containing a 3' U residue were found preferentially, suggesting that the 3' U residue might be able to undergo base pairing with the terminal 5' A residue of the vRNA during transcription initiation. This would be in good agreement with previous observations in orthobunyaviruses and hantaviruses, where capped primers preferentially terminate at G residues, potentially facilitating RNA primer binding to the terminal 5' C residue (Garcin *et al.*, 1995). As observed for orthobunyaviruses, a number of primer sequences contained 3' GU or 3' AGU residues (Bouloy *et al.*, 1990).

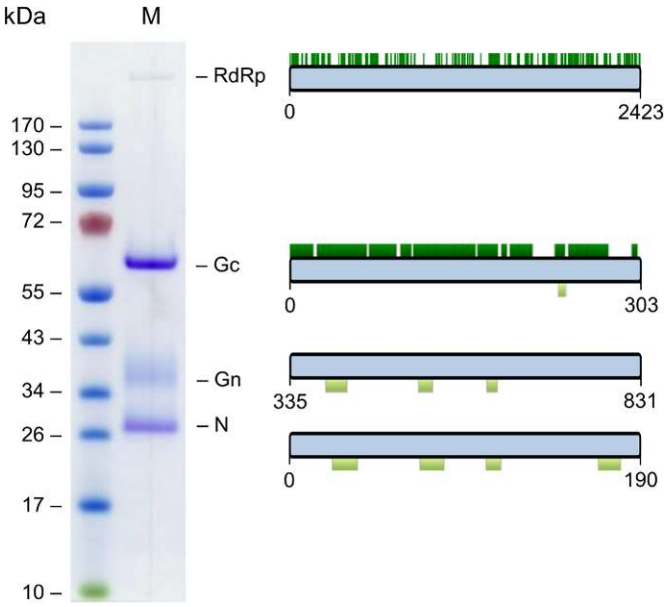

Figure II.8 SDS-PAGE analysis of HEBV major structural proteins. Particles were purified from cell culture supernatants of infected C6/36 cells by gradient ultracentrifugation. Proteins were stained with Coomassie blue R-250. Obtained MALDI-TOF data are shown below and LC-MS data are shown above the schematic view of proteins to the right.

Analyses of RNA products in infected cells indicated that HEBV and KIBV generate truncated mRNAs, similar to what has been described for other bunyaviruses such as snowshoe hare virus, an orthobunyavirus whose S segment mRNA is about 85 nt shorter than the vRNA species (Eshita *et al.*, 1985).

Taken together, our findings suggest that HEBV, TAIV, and KIBV cannot be assigned to any existing bunyavirus genus, while they share common features with each other sufficient to classify them as one genus. Although they are somewhat more closely related to orthobunyaviruses than to other bunyavirus genera, their genome organization and phylogenetic relationships separate them from other genera. Further studies, particularly on their host restriction and antigenic properties, will be necessary to support their putative classification into a separate novel genus.

Chapter III

No Evidence of Gouléako and Herbert Virus Infections in Pigs, Côte d'Ivoire and Ghana

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III.1 INTRODUCTION

A recent report suggested that 2 novel bunyaviruses discovered in insects in Côte d'Ivoire caused lethal disease in swine in South Korea. We conducted cell culture studies and tested serum from pigs exposed to mosquitoes in Côte d'Ivoire and Ghana and found no evidence for infection in pigs.

Orthobunyaviruses and phleboviruses are transmitted to animals and humans by blood-feeding arthropods such as mosquitoes, sandflies, and ticks (Elliott & Brennan, 2014; Elliott, 2014). Infection can cause systemic disease, including encephalitis or hemorrhagic fevers. Members of both genera of viruses encode a nonstructural (NS) protein that suppresses the antiviral interferon response of the vertebrate host (Bouloy *et al.*, 2001; Bridgen *et al.*, 2001). We recently discovered 2 novel prototypic bunyaviruses in mosquitoes in Côte d'Ivoire (Marklewitz *et al.*, 2011, 2013). Named Gouléako virus (GOLV) and Herbert virus (HEBV), the viruses tentatively define 2 novel bunyavirus-family genera that are in a sister relationship to the genera *Phlebovirus* and *Orthobunyavirus*, respectively. Neither virus encodes NS proteins, nor do the viruses infect vertebrate cells or cause disease in mice that have been intracerebrally inoculated with the viruses (Auguste *et al.*, 2014; Marklewitz *et al.*, 2011, 2013). Replication of both viruses is blocked at temperatures above 31°C, suggesting that the viruses are unlikely to infect mammals (Marklewitz *et al.*, 2015).

Chung et al. recently reported that, in 2013, GOLV and HEBV caused prevalent and lethal infections in swine in South Korea (Chung *et al.*, 2014). In that study, >500 pigs from 40 farms were tested for both viruses, and viral RNA was detected in up to 79% of diseased and 55% of healthy pigs. Dead pigs carried virus in their lungs and intestines. GOLV was isolated from swine serum in porcine kidney 15 cells. These results suggest the discovery of disease caused by these 2 novel viruses in a major livestock species. Because of the implications of this finding, we attempted verification.

III.2 THE STUDY

We first extended our recent cell culture studies to include porcine kidney 15 and human embryonic kidney 293 cells, which were the type of cells used by Chung et al. (Chung *et al.*, 2014). Human hepatocellular 7 carcinoma cells were also included because they are highly susceptible to virus infection, as are Vero cells and several other cell lines we used in earlier studies (Chung *et al.*, 2014; Marklewitz *et al.*, 2013). Infections with GOLV and HEBV were performed at multiplicities of infection of 1 in doublets in all cell lines. Vesicular stomatitis virus was used as a positive control at multiplicity of infection 1. Cell culture supernatants were analyzed for viral RNA after 0, 3, and 6 days by real-time RT-PCR (Marklewitz *et al.*, 2011, 2013). No replication of GOLV and HEBV was detected, whereas vesicular stomatitis virus replicated to high concentrations [Figure 1]. Three blind passages on fresh cells failed to yield virus.

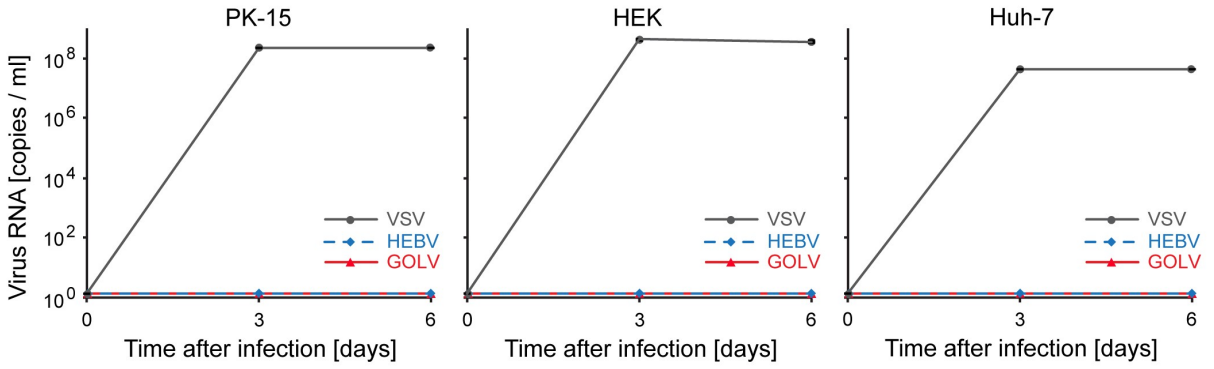

Figure III.1 Infection of cells with Vesicular stomatitis virus (VSV), Herbert virus (HEBV), and Gouléako virus (GOLV). A) Porcine kidney 15 cells; B) human embryonic kidney cells; C) human hepatocellular 7 cells. Cells were infected at a multiplicity of infection of 1. The number of viral genome copies in cell culture supernatants were measured at 0, 3, and 6 days postinfection by real-time reverse transcription PCR.

Because cell culture experiments may not show the full host range of a specific virus, we tested serum samples collected in 2008 from *Sus scrofa domestica* pigs in Gouléako, the rural village where GOLV and HEBV were first isolated from mosquitoes in Côte d'Ivoire (Marklewitz *et al.*, 2011, 2013). The 28 tested samples represented nearly all the pigs kept in Gouléako at that time, all of which were constantly exposed to mosquitoes. We also tested 108 serum samples collected in 2011 from mosquito-exposed swine in Kumasi, Ghana, where mosquitoes were found to be infected with HEBV (Marklewitz *et al.*, 2013) and GOLV (S. Junglen, unpublished data).

All samples were tested for virus by real-time RT-PCR (Marklewitz *et al.*, 2011, 2013) and tested for antibodies against GOLV and HEBV nucleocapsid proteins by recombinant immunofluorescence assay (Meyer *et al.*, 2014). All samples were negative for the viruses [Table III.1]. Figure III.2 shows antigen controls and results from 1 representative swine serum sample.

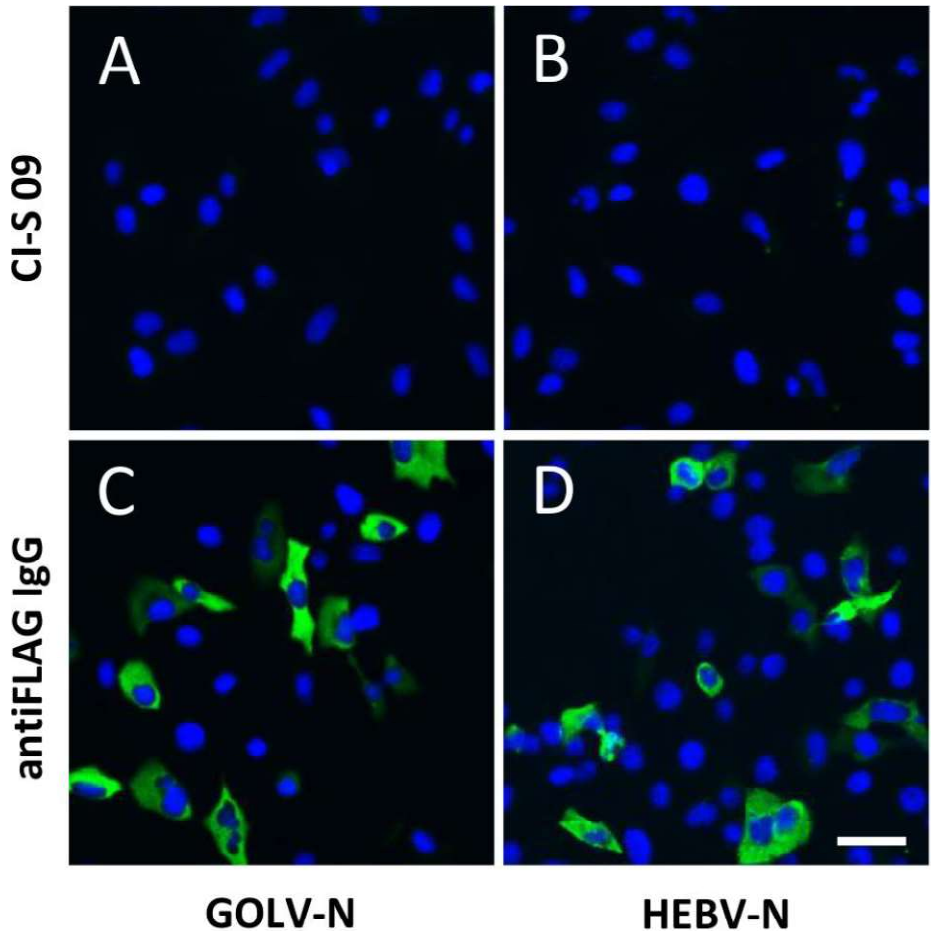

Figure III.2 Immunofluorescence patterns for antibodies against Gouléako virus (GOLV) and Herbert virus (HEBV) in serum samples from swine, Côte d'Ivoire (CI), 2008, and Ghana, 2011. Figure shows representative results from 1 pig (labeled CI-S 09) from which serum was tested against overexpressed recombinant nucleocapsid protein of A) GOLV and B) HEBV in VeroB4 cells. Anti-FLAG IgG antibodies were used to control for overexpression of C) GOLV-nucleocapsid (N) and D) HEBV-N. Scale bar indicates 20 µm. All photographs were taken at equivalent exposure settings.

To compare the viruses found in pigs in South Korea with viruses found in mosquitoes in Africa, we replicated methods used by Chung et al. (Chung *et al.*, 2014) and amplified a region of the GOLV glycoprotein precursor gene from 27 GOLV strains in mosquitoes [refer to II.3 Material & Methods]. Nucleotide sequence distance among mosquito strains was as high as 9.0%. The viruses found in the pigs fell within the genetic diversity of viral strains of GOLV and HEBV and did not constitute phylogenetic outliers [Figure III.3, panel A]. The analyzed fragment had 6 aa exchanges, but they were insufficient for drawing conclusions about protein function because the fragment did not include domains putatively relevant for receptor binding [Supplement Figure III.1].

Table III.1 Porcine serum samples tested for infection with GOLV and HEBV by using real-time reverse–transcription PCR and rIFA, in Côte d'Ivoire and Ghana, 2008–2011*

					rIFA		Viral RNA	
Sample ID	Sex	Age, mo*	Origin**	Year	GOLV	HEBV	GOLV	HEBV
CI-S 01	Male	12	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 02	Female	12	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 03	Female	12	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 04	Male	11	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 05	Female	9	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 06	Female	3	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 07	Female	3	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 08	Male	3	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 09	Female	3	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 10	Female	6	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 11	Female	3	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 12	Female	3	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 13	Female	12	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 14	Male	3	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 15	Female	8	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 16	Female	4	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 17	Female	4	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 18	Female	2	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 19	Male	2	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 20	Female	4	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 21	Male	4	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 22	Female	2	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 23	Female	3	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 24	Female	2	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 25	Female	3	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 26	Female	3	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 27	Female	3	Gouléako, CI	2008	Neg	Neg	Neg	Neg
CI-S 28	Female	5	Gouléako, CI	2008	Neg	Neg	Neg	Neg
GH-S 01	Female	24	Amanfrom, GH	2011	Neg	Neg	Neg	Neg
GH-S 02	Female	6	Amanfrom, GH	2011	Neg	Neg	Neg	Neg
GH-S 04	Male	6	Amanfrom, GH	2011	Neg	Neg	Neg	Neg
GH-S 05	Female	36	Amanfrom, GH	2011	Neg	Neg	Neg	Neg
GH-S 06	Male	6	Amanfrom, GH	2011	Neg	Neg	Neg	Neg
GH-S 07	Male	6	Amanfrom, GH	2011	Neg	Neg	Neg	Neg
GH-S 08	Female	8	Amanfrom, GH	2011	Neg	Neg	Neg	Neg
GH-S 09	Female	8	Amanfrom, GH	2011	Neg	Neg	Neg	Neg
GH-S 10	Female	7	Amanfrom, GH	2011	Neg	Neg	Neg	Neg
GH-S 11	Female	7	Amanfrom, GH	2011	Neg	Neg	Neg	Neg
GH-S 12	Female	1.5	Sokoban New Town, GH	2011	Neg	Neg	Neg	Neg
GH-S 13	Male	6	Dompoase, GH	2011	Neg	Neg	Neg	Neg
GH-S 14	Male	8	Dompoase, GH	2011	Neg	Neg	Neg	Neg
GH-S 15	Male	6	Dompoase, GH	2011	Neg	Neg	Neg	Neg
GH-S 16	Female	1.5	Sokoban New Town, GH	2011	Neg	Neg	Neg	Neg
GH-S 17	Female	6	Sokoban New Town, GH	2011	Neg	Neg	Neg	Neg
GH-S 18	Male	1.5	Sokoban New Town, GH	2011	Neg	Neg	Neg	Neg
GH-S 19	Male	6	Sokoban New Town, GH	2011	Neg	Neg	Neg	Neg
GH-S 20	Male	6	Sokoban New Town, GH	2011	Neg	Neg	Neg	Neg
GH-S 21	Female	8	Dompoase, GH	2011	Neg	Neg	Neg	Neg
GH-S 22	Male	6	Dompoase, GH	2011	Neg	Neg	Neg	Neg
GH-S 23	Male	6	Dompoase, GH	2011	Neg	Neg	Neg	Neg
GH-S 24	Male	6	Dompoase, GH	2011	Neg	Neg	Neg	Neg

GH-S 25	Female	6	Dompoase, GH	2011	Neg	Neg	Neg	Neg
GH-S 26	Male	8	Dompoase, GH	2011	Neg	Neg	Neg	Neg
GH-S 27	Female	6	Dompoase, GH	2011	Neg	Neg	Neg	Neg
GH-S 28	Female	8	Dompoase, GH	2011	Neg	Neg	Neg	Neg
GH-S 31	Male	5	Dompoase, GH	2011	Neg	Neg	Neg	Neg
GH-S 32	Male	5	Dompoase, GH	2011	Neg	Neg	Neg	Neg
GH-S 33	Female	5	Akropong, GH	2011	Neg	Neg	Neg	Neg
GH-S 34	Male	5	Akropong, GH	2011	Neg	Neg	Neg	Neg
GH-S 35	Male	5	Akropong, GH	2011	Neg	Neg	Neg	Neg
GH-S 36	Male	5	Akropong, GH	2011	Neg	Neg	Neg	Neg
GH-S 37	Female	5	Akropong, GH	2011	Neg	Neg	Neg	Neg
GH-S 38	Female	7	Akropong, GH	2011	Neg	Neg	Neg	Neg
GH-S 39	Female	7	Akropong, GH	2011	Neg	Neg	Neg	Neg
GH-S 40	Male	6	Akropong, GH	2011	Neg	Neg	Neg	Neg
GH-S 41	Male	6	Akropong 2, GH	2011	Neg	Neg	Neg	Neg
GH-S 42	Male	6	Akropong 2, GH	2011	Neg	Neg	Neg	Neg
GH-S 43	Male	6	Akropong, GH	2011	Neg	Neg	Neg	Neg
GH-S 44	Female	6	Akropong, GH	2011	Neg	Neg	Neg	Neg
GH-S 45	Female	6	Akropong, GH	2011	Neg	Neg	Neg	Neg
GH-S 46	Male	5	Akropong, GH	2011	Neg	Neg	Neg	Neg
GH-S 47	Female	5	Akropong, GH	2011	Neg	Neg	Neg	Neg
GH-S 48	Male	5	Akropong, GH	2011	Neg	Neg	Neg	Neg
GH-S 49	Female	5	Akropong, GH	2011	Neg	Neg	Neg	Neg
GH-S 50	Female	6	Akropong 2, GH	2011	Neg	Neg	Neg	Neg
GH-S 51	Female	6	Akropong 2, GH	2011	Neg	Neg	Neg	Neg
GH-S 52	Male	6	Akropong 2, GH	2011	Neg	Neg	Neg	Neg
GH-S 53	Male	7	Akropong 2, GH	2011	Neg	Neg	Neg	Neg
GH-S 54	Male	7	Akropong 2, GH	2011	Neg	Neg	Neg	Neg
GH-S 55	Female	7	Akropong 2, GH	2011	Neg	Neg	Neg	Neg
GH-S 56	Female	7	Akropong 2, GH	2011	Neg	Neg	Neg	Neg
GH-S 57	Male	7	Akropong 2, GH	2011	Neg	Neg	Neg	Neg
GH-S 58	Male	5	Essienimpong, GH	2011	Neg	Neg	Neg	Neg
GH-S 59	Female	6	Essienimpong, GH	2011	Neg	Neg	Neg	Neg
GH-S 60	Male	5	Essienimpong, GH	2011	Neg	Neg	Neg	Neg
GH-S 61	Male	5	Essienimpong, GH	2011	Neg	Neg	Neg	Neg
GH-S 62	Male	4	Akropong 2, GH	2011	Neg	Neg	Neg	Neg
GH-S 63	Female	4	Akropong 2, GH	2011	Neg	Neg	Neg	Neg
GH-S 64	Male	4	Akropong 2, GH	2011	Neg	Neg	Neg	Neg
GH-S 65	Female	4	Akropong 2, GH	2011	Neg	Neg	Neg	Neg
GH-S 66	Female	4	Akropong 2, GH	2011	Neg	Neg	Neg	Neg
GH-S 67	Male	5	Essienimpong, GH	2011	Neg	Neg	Neg	Neg
GH-S 68	Female	5	Essienimpong, GH	2011	Neg	Neg	Neg	Neg
GH-S 69	Female	5	Essienimpong, GH	2011	Neg	Neg	Neg	Neg
GH-S 70	Female	5	Essienimpong, GH	2011	Neg	Neg	Neg	Neg
GH-S 71	Female	6	Essienimpong, GH	2011	Neg	Neg	Neg	Neg
GH-S 72	Female	6	Essienimpong, GH	2011	Neg	Neg	Neg	Neg
GH-S 73	Female	8	Onwe, GH	2011	Neg	Neg	Neg	Neg
GH-S 74	Female	8	Onwe, GH	2011	Neg	Neg	Neg	Neg
GH-S 75	Female	8	Onwe, GH	2011	Neg	Neg	Neg	Neg
GH-S 76	Male	8	Onwe, GH	2011	Neg	Neg	Neg	Neg
GH-S 77	Male	8	Onwe, GH	2011	Neg	Neg	Neg	Neg
GH-S 78	Female	5	Onwe, GH	2011	Neg	Neg	Neg	Neg
GH-S 79	Male	8	Onwe, GH	2011	Neg	Neg	Neg	Neg
GH-S 80	Female	8	Onwe, GH	2011	Neg	Neg	Neg	Neg
GH-S 81	Female	5	Onwe, GH	2011	Neg	Neg	Neg	Neg

GH-S 82	Female	5	Onwe, GH	2011	Neg	Neg	Neg	Neg
GH-S 83	Female	7	Ejisu Krapa, GH	2011	Neg	Neg	Neg	Neg
GH-S 84	Female	7	Ejisu Krapa, GH	2011	Neg	Neg	Neg	Neg
GH-S 85	Female	7	Ejisu Krapa, GH	2011	Neg	Neg	Neg	Neg
GH-S 86	Female	7	Ejisu Krapa, GH	2011	Neg	Neg	Neg	Neg
GH-S 87	Male	7	Ejisu Krapa, GH	2011	Neg	Neg	Neg	Neg
GH-S 89	Female	5	Ejisu Krapa, GH	2011	Neg	Neg	Neg	Neg
GH-S 90	Female	5	Ejisu Krapa, GH	2011	Neg	Neg	Neg	Neg
GH-S 91	Male	4	Ejisu Krapa, GH	2011	Neg	Neg	Neg	Neg
GH-S 92	Female	5	Ejisu Krapa, GH	2011	Neg	Neg	Neg	Neg
GH-S 93	Female	12	Abattoir, GH	2011	Neg	Neg	Neg	Neg
GH-S 94	Female	12	Abattoir, GH	2011	Neg	Neg	Neg	Neg
GH-S 95	Female	12	Abattoir, GH	2011	Neg	Neg	Neg	Neg
GH-S 96	Male	12	Abattoir, GH	2011	Neg	Neg	Neg	Neg
GH-S 97	Male	12	Abattoir, GH	2011	Neg	Neg	Neg	Neg
GH-S 98	Female	12	Abattoir, GH	2011	Neg	Neg	Neg	Neg
GH-S 99	Male	12	Abattoir, GH	2011	Neg	Neg	Neg	Neg
GH-S 100	Female	12	Abattoir, GH	2011	Neg	Neg	Neg	Neg
GH-S 101	Female	12	Abattoir, GH	2011	Neg	Neg	Neg	Neg
GH-S 102	Male	12	Abattoir, GH	2011	Neg	Neg	Neg	Neg
GH-S 103	Female	12	Abattoir, GH	2011	Neg	Neg	Neg	Neg
GH-S 104	Female	5	Abattoir, GH	2011	Neg	Neg	Neg	Neg
GH-S 105	Female	12	Abattoir, GH	2011	Neg	Neg	Neg	Neg
GH-S 106	Female	4	Abattoir, GH	2011	Neg	Neg	Neg	Neg
GH-S 107	Male	12	Abattoir, GH	2011	Neg	Neg	Neg	Neg
GH-S 108	Female	12	Abattoir, GH	2011	Neg	Neg	Neg	Neg

**CI, Côte d'Ivoire; GH, Ghana; GOLV, Gouléako virus; HEBV, Herbert virus; Neg, Negative results.

*Age is age of pig from which serum sample was collected.

Small RT-PCR fragments from the RdRp gene were presented by Chung et al. for HEBV. We performed phylogenetic analyses to compare these swine-derived sequences with sequences from all mosquito-derived viruses from which we could sequence the corresponding genome region [Figure III.3, panel B]. Comparison of swine-derived sequences with the phylogeny of mosquito-derived HEBV strains, constructed on the basis of the third conserved region of the RdRp [Figure III.3, panel C], showed that the strains from South Korea fell within the phylogenetic diversity of HEBV strains identified in West Africa. Supplement Figure III.2 shows nucleotide- and amino acid-based alignments.

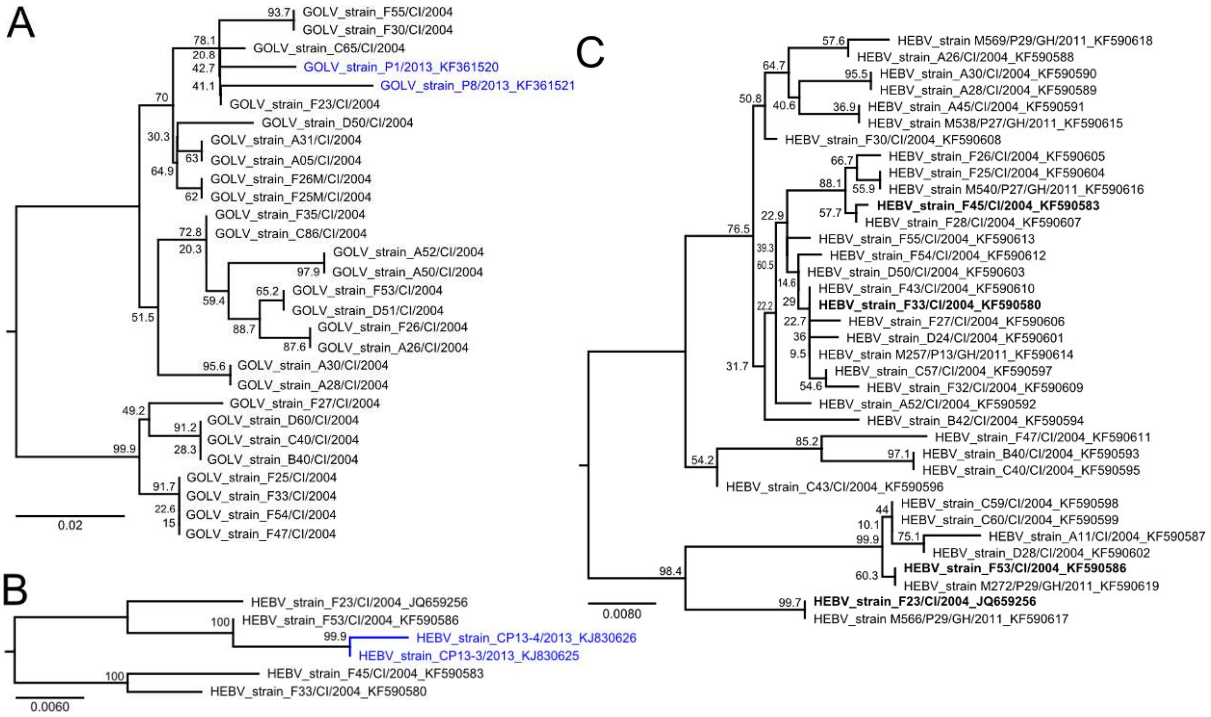

Figure III.3 Maximum-likelihood phylogenetic analyses of Gouléako virus (GOLV) and Herbert virus (HEBV) strains from mosquitoes in Côte d'Ivoire, 2004, and Ghana, 2011, and virus strains detected by Chung el al. (*9*) in pigs in South Korea. A) Analysis of the glycoprotein precursor gene of GOLV strains identified in mosquitoes collected in Côte d'Ivoire and Ghana and of strains detected in swine in South Korea. Sequences originating from swine are shown in bold. B) Analysis of the RNA-dependent RNA polymerase gene of HEBV strains from mosquitoes and swine. Sequences originating from swine are shown in bold. C) Analysis of all identified HEBV strains found in mosquitoes. HEBV strains used for phylogenetic analyses in panel B are shown in bold. GOLV strains F25M/CI/2004 and F26/CI/2004 were found in male mosquitoes. Scale bar indicates nucleotide substitutions per position in the alignment.

III.3 MATERIAL & METHODS

III.3.1 Growth Kinetics

Porcine kidney (PK)-15 cells, human embryonic kidney (HEK)-293 cells, and human hepatocellular carcinoma (HuH)-7 cells were infected with GOLV, HEBV, and vesicular stomatitis virus (VSV) as a positive control at MOI of 1, as described (Elliott & Brennan, 2014; Elliott, 2014). Cell culture supernatants were analyzed for viral genome copy numbers at 0, 3, and 6 days postinfection by real-time reverse transcription PCR (Elliott & Brennan, 2014; Elliott, 2014).

III.3.2 Amplification of GOLV Glycoprotein Precursor Gene Sequences

RNA was extracted from infected C6/36 cells by using the Viral RNA Kit (QIAGEN, Hilden, Germany) and cDNA synthesis was performed by using SuperScript III (Thermo Fisher Scientific, Lithuania) Glycoprotein precursor gene fragments were amplified by using primers based on strain GOLV/A5/CI/2005 and Platinum Taq polymerase, according to the manufacturer's instructions (Thermo Fisher Scientific, Lithuania). PCR products were analyzed by agarose gel electrophoresis and sequenced by Seqlab (Göttingen, Germany).

Sequences were deposited in the GenBank database (National Center for Biotechnology Information, Bethesda, MD, USA) under accession number KT387771–KT387796.

III.3.3 Phylogenetic Analyses

GOLV glycoprotein precursor gene and HEBV RdRp sequences were aligned by using the multiple sequence alignment program MAFFT (http://wiki.hpc.ufl.edu/doc/PhyML); maximum likelihood analyses were inferred by using PhyML (https://code.google.com/p/phyml/) with the HKY85 substitution matrix and 1,000 bootstrap replicates in Geneiuos (Biomatters, Aukland, New Zealand; http://www.geneious.com/).

III.3.4 PCR Screening of Swine Serum Samples

Ethical review and clearances of animal handling procedure were obtained from the Ghana Forestry Commission of the Ministry of Food and Agriculture. RNA was extracted from 15µL of porcine serum samples mixed with 55µL Dulbecco's Phosphate-Buffered Saline by using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). Random cDNA synthesis was performed by using SuperScript III (Thermo Fisher Scientific, Lithuania). Viral genome copies were measured by real-time reverse–transcription PCR, as described previously (Elliott & Brennan, 2014; Elliott, 2014). The Table III.1 shows samples tested and results.

III.3.5 Recombinant Nucleocapsid Immunofluorescence Assay (IFA)

Porcine serum samples were screened for presence of antibodies against the GOLV and HEBV viruses in 1:20 dilutions by rIFA as described (Bridgen *et al.*, 2001). C-terminally FLAG-tagged full nucleocapsid genes of GOLV or HEBV were amplified from cDNA by using the primers GOLV-N-XbaI-F (5'-GCTCTAGAGCCACCATGGCAACAGTTACTCAGAATGACATTCAG), GOLV-N-FLAG-C-XbaI-R (5'-GCTCTAGATCACTTGTCATCGTCGTCCTTGTAGTCACCAGCTTCCA-TCAGTTTTCCGGCCGC), HEBV-N-BamHI-F (5'-CGGGATCCGCCACCATGGCTACCAATTTTGAAT-TCAATGATAAC), and HEBV-N-FLAG-C-SphI-R (5'-ACATGCATGCTCACTTGTCATCGTCGTCCTTG-TAGTCACCAGCTTGAGGCCATATTTTGTTGATCAGTG). The amplified genes were then cloned into a pCG1 eukaryotic expression vector.

Plasmids were sequence confirmed. Transfected cells were used in indirect immunofluorescence assays with goat anti-swine IgG-Alexa Fluor 488 conjugate (Sigma, St. Louis, USA) in 1:200 dilution for detection of bound swine serum antibody. A rabbit anti-FLAG antibody and goat anti-rabbit fluorescein-labeled conjugate in 1:200 dilution (Dianova, Hamburg, Germany) were used to confirm expression of viral proteins. A c-terminal flag tag will be expressed only when the upstream viral protein ORF is intact. Cell nuclei were stained with ProLong Gold Antifade Mountant (Thermo Fisher Scientific, Lithuania) with DAPI (4', 6-diamidino-2-phynylindole).

III.4 CONCLUSIONS

Our results contrast with those of Chung et al. (Chung *et al.*, 2014) for several possible reasons. First, the viruses infecting swine in South Korea may constitute variants of GOLV and HEBV that can infect vertebrates. The presence of an NSs protein in phleboviruses and orthobunyaviruses provides interferon resistance required to infect vertebrates efficiently (Bouloy *et al.*, 2001; Bridgen *et al.*, 2001). Because full genome sequences from swine viruses detected by Chung et al. are not available, we have no information on the presence of NS proteins in these viruses. Furthermore, our detection assays might have failed to detect variant viruses. However, our RT-PCR assays have been shown to detect variant viruses, have been validated for sensitivity (≈100 viral genome copies per mL in liquid specimens), and provide high specificity by probe detection (Marklewitz et al., 2011, 2013). A concern regarding the results of Chung et al. is the use of RT-PCR assays based on SYBR Green (Thermo Fisher Scientific, Lithuania) product detection, which, from our experience, is prone to yield nonspecific results because no probe is used in this assay. Nevertheless, RT-PCR products in Chung et al. have been confirmed by sequencing. Some sequences presented by these researchers contained stop codons in the HEBV RdRp and the GOLV glycoprotein precursor genes, making it unlikely that these sequences represent replicating viruses. Besides technical explanations, these sequences could represent viral genome fragments integrated in genomes of organisms, such as insects, that are eaten by pigs in the region. Integration of RNA virids derived from flaviviruses into the host genome has been described in insects (Crochu *et al.*, 2004). Testing food eaten by swine for insect DNA or viral RNA could yield insight. In addition, we may have collected serum when no active virus infections occurred in tested animals. However, past infections would have been shown by antibody tests. Because bunyaviruses from all vertebrate-infecting genera induce antibodies against the nucleoprotein (Ergunay *et al.*, 2014; Lazutka *et al.*, 2014; Williams *et al.*, 2011), we are confident about our choice of antigen in our assays. Chung et al. presented no serologic results to support virus detections (Chung *et al.*, 2014).

Several technical issues in the study by Chung et al. should be clarified further. First, RNA concentration in tissue, as determined by RT-PCR, did not correlate with the success of probe-based immunohistochemistry in several organ samples (Chung *et al.*, 2014). Second, supernatants from the virus isolate from South Korea showed high cytopathogenic activity in cell culture (103–105 cytopathogenic units/ml) but low levels of concomitant viral RNA by RT-PCR. Because no antigen detection in cells was attempted, the cytopathogenic effect could have been caused by any other virus blindly isolated. One of the most infectious and deadly swine pathogens, the porcine reproductive and respiratory syndrome virus (Chand *et al.*, 2012), was co-detected in lung samples of dead pigs in South Korea (Chung *et al.*, 2014).

The finding of genome fragments of GOLV and HEBV in swine in South Korea needs to be more fully explored. However, with no further independent proof of infection of swine or other vertebrates, HEBV and GOLV should not be considered epizootic pathogens or arboviruses.

Chapter IV

Evolutionary and phenotypic analysis of live virus isolates suggests arthropod origin of a pathogenic RNA virus family

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IV.1 INTRODUCTION

The evolutionary origins of arboviruses are unknown because their typical dual host tropism is paraphyletic within viral families. Here we studied one of the most diversified and medically relevant RNA virus families, the *Bunyaviridae*, in which four of five established genera are transmitted by arthropods. We define two cardinally novel bunyavirus groups based on live isolation of 26 viral strains from mosquitoes (Jonchet virus [JONV], eight strains; Ferak virus [FERV], 18 strains). Both viruses were incapable of replicating at vertebratetypical temperatures but replicated efficiently in insect cells. Replication involved formation of vRNA and mRNA, including cap-snatching activity. SDS/PAGE, mass spectrometry, and Edman degradation identified translation products corresponding to virion-associated RdRp, glycoprotein precursor protein, glycoproteins Gn and Gc, as well as putative nonstructural proteins NSs and NSm. Distinct virion morphologies suggested ancient evolutionary divergence, with bunyavirus-typical morphology for FERV (spheres of 60–120 nm) as opposed to an unusual bimorphology for JONV (tubular virions of 60 × 600 nm and spheres of 80 nm). Both viruses were genetically equidistant from all other bunyaviruses, showing <15% amino acid identity in the RdRp palm domain. Both had different and unique conserved genome termini, as in separate bunyavirus genera. JONV and FERV define two novel sister taxons to the superclade of orthobunyaviruses, tospoviruses, and hantaviruses. Phylogenetic ancestral state reconstruction with probabilistic hypothesis testing suggested ancestral associations with arthropods at deep nodes throughout the bunyavirus tree. Our findings suggest an arthropod origin of bunyaviruses.

IV.1.1 Significance

Knowledge of the origin and evolution of viruses provides important insight into virus emergence involving the acquisition of genes necessary for the infection of new host species or the development of pathogenicity. The family *Bunyaviridae* contains important arthropod-borne pathogens of humans, animals, and plants. In this study, we provide a comprehensive characterization of two novel lineages of insect-specific bunyaviruses that are in basal phylogenetic relationship to the rodent-borne hantaviruses, the only genus within the *Bunyaviridae* that is not transmitted by arthropod vectors. These data, together with ancestral state reconstruction of bunyavirus hosts formajor virus lineage bifurcations, suggest that the vertebrate-infecting viruses evolved from arthropod-specific progenitors.

IV.2 THE STUDY

Arboviruses are viruses with dual host tropism that are transmitted to their vertebrate hosts during the arthropod host's blood-feeding. Arboviruses are found in several different RNA virus families as well as in a single DNA virus family, suggesting the dual host tropism has evolved by convergence. However, it is unclear whether arboviruses stem from arthropods

or vertebrates, as dual host tropism is a paraphyletic property. All families of interest contain additional taxa with monotropism for either arthropods or vertebrates. Only for the genus Flavivirus within the family *Flaviviridae* has an evolution from insect-specific viruses been suggested, as new insect-specific flaviviruses have recently been discovered that branch deeper than congeneric arboviruses (Cook *et al.*, 2012). Here we studied the case of one of the most genetically diversified families of RNA viruses, the family *Bunyaviridae* (Plyusnin *et al.*, 2012).

Bunyaviruses contain important pathogens of humans, livestock, and plants. With the exception of the rodent-borne hantaviruses, all bunyaviruses are transmitted by arthropod vectors (Schmaljohn & Nichol, 2007). In addition to the five established genera, we have recently described two novel groups of putatively insect-specific bunyaviruses isolated from mosquitoes (Marklewitz *et al.*, 2011, 2013). One clade, defined by the type species GOLV (Marklewitz *et al.*, 2011), shares old common ancestors with all members of the genus *Phlebovirus*, and the second clade, defined by HEBV, TAIV, and KIBV (Marklewitz *et al.*, 2013), branches from a deep node in sister relationship to the genus *Orthobunyavirus*. Both virus groups have been proposed to constitute novel bunyavirus genera on the basis of their phylogenetic positions and other criteria such as serological distinction and differences in genome composition, including the absence of NSs and NSm proteins, as well as the lengths and sequences of conserved noncoding elements at genome segment termini. More recently, bona fide bunyavirus sequences distant from all described bunyaviruses were detected in phantom midges as well as in transcriptomes from other insects (Ballinger *et al.*, 2014). However, corresponding viruses could not be isolated in cell culture, leaving doubts about whether these virids represent extant viruses.

To further examine the diversity of bunyaviruses, we screened cytopathic *Aedes albopictus* cell cultures inoculated with mosquitoes from the same region as GOLV and HEBV in Côte d'Ivoire (Junglen *et al.*, 2009a). As a new approach, we used sensitivity to temperature to differentiate between insect-specific viruses and arboviruses. Our findings enable a reconciliation of the origin and evolution of the family *Bunyaviridae*.

IV.3 RESULTS

IV.3.1 Virus Isolation and Morphology

Two viruses, designated Jonchet virus (JONV) and Ferak virus (FERV), were isolated in C6/36 cells from mosquitoes collected in the Taï National Park region, Côte d'Ivoire (sampling described in Junglen *et al.*, 2009a). Both viruses induced strong but distinct CPE 4–5 dpi. JONV induced extensive syncytia formation, with some cells showing stretching and filamentous extensions [Supplement Figure IV.1A]. Cells infected with FERV showed stretching and tapering with slight aggregation and very sporadic formation of syncytia [Supplement Figure IV.1B]. Viral particles were purified by gradient ultracentrifugation from infected cell culture supernatants and examined by electron microscopy. The spherical, enveloped FERV virions were pleomorphic, with a diameter of 60–120 nm [Figure IV.1B],

morphologically resembling GOLV and HEBV (Marklewitz *et al.*, 2011, 2013). Two types of enveloped virions were associated with JONV. Virions either had a tubular morphology of about 60 nm × up to 600 nm or were spherical with a diameter of about 80 nm [Figure IV.1A]. Both types of JONV virions co-occurred regularly in ultrathin sections of JONV-infected C6/36 cells in all independent cell culture isolates, but not in noninfected cells and not in cells infected with FERV, suggesting that both forms belong to one virus [Figure IV.1C].

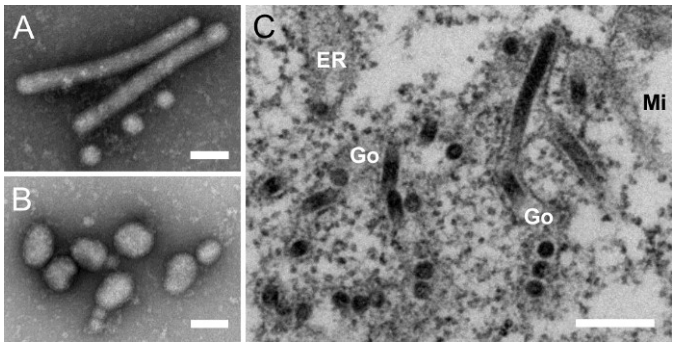


Figure IV.1 JONV and FERV morphology. (A and B) Negative-stained virions of JONV (A) and FERV (B) sedimented by ultracentrifugation. (C) Ultrathin sections of JONV-infected C6/36 cells. Mi, mitochondria; Go, Golgi apparatus; ER, endoplasmatic reticulum. (Scale bars, 100 nm in A and B and 250 nm in C.)

IV.3.2 Genome Organization and Phylogenetic Analyses

Entire prototype genomes of JONV and FERV were initially determined from infectious cell culture supernatants. RT-PCR on additional CPEpositive cell cultures inoculated with mosquito pools identified a further seven isolates of JONV and 17 isolates of FERV [Figure IV.3B]. Phylogenetic analysis of sequenced RT-PCR products showed segregation into three clades for FERV and at least two distinct clades for the JONV isolates [Supplement Figure IV.2 A and B]. To determine the purity of viral isolates, the coding regions of three JONV and FERV isolates each were sequenced by next-generation sequencing. Pairwise nt identities among JONV and FERV isolates were higher than 95.9% and 91.6%, respectively. Because of the high similarity in genome organization among isolates, only one prototype genome per virus is described here.

JONV and FERV prototypes were highly distinct from each other and from all other bunyaviruses. The JONV genome consisted of three segments of 1,745 nt (S), 5,449 nt (M), and 6,904 nt (L) [Figure IV.2A]. The FERV genome was shorter and comprised three segments of 1,527 nt (S), 4,274 nt (M), and 6,938 nt (L) [Figure IV.2D]. Pairwise aa identities between JONV and FERV ORFs were 22.1%, 10.3%, and 23.8% for S, M, and L segments, respectively. There were seven reverse-complementary terminal nucleotides in JONV genome segments (5’-AGUAGUA), and 11 in FERV genome segments (5’-AGUAGUAAACA). These conserved terminal sequences were different from those of any other bunyaviruses. However, six of seven terminal nucleotides shared between JONV and FERV were also present in the recently described novel clade of bunyaviruses defined by HEBV (Marklewitz *et al.*, 2013), as well as in all members of the genus *Orthobunyavirus* (5’-AGUAGU) (Plyusnin *et al.*, 2012), suggesting a common origin.

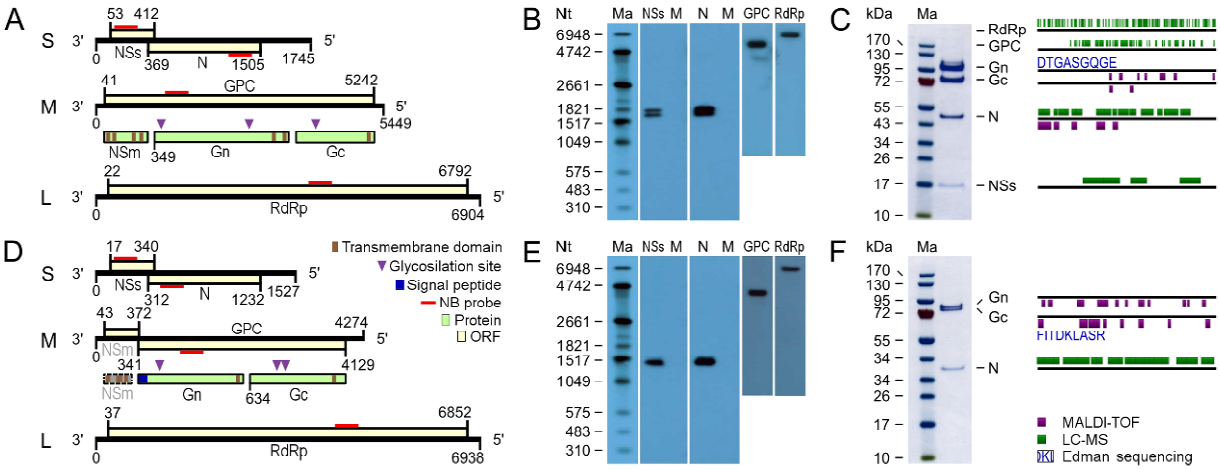


Figure IV.2 JONV and FERV genome organization, replication, and expression. (A and D) Schematic diagram of JONV (A) and FERV (D) genomes. (B and E) Northern blot analyses of JONV (B) and FERV (E) RNAs in infected C6/36 cells. RNA from noninfected C6/36 cells was used as mock control (M). (C and F) Major structural proteins of JONV (C) and FERV (F) analyzed by SDS/PAGE, using virions purified by gradient ultracentrifugation.

JONV and FERV L segments comprisedORFs of 2,256 and 2,271 aa in cRNA sense, respectively. Putative 256- and 262-kDa proteins most likely represented the RdRp proteins. Low similarities (identity <15%) in parts of RdRp protein sequences of bunyaviruses from all genera as well as tenuiviruses were only identified on the protein level. Similarities were only identified for the region $_{603}$LVIN-LFGY$_{1287}$ of JONV and $_{440}$SQLH-PKYI$_{1254}$ of FERV, which correspond to the third conserved region of the bunyavirus RdRp protein [Supplement Figure IV.3].

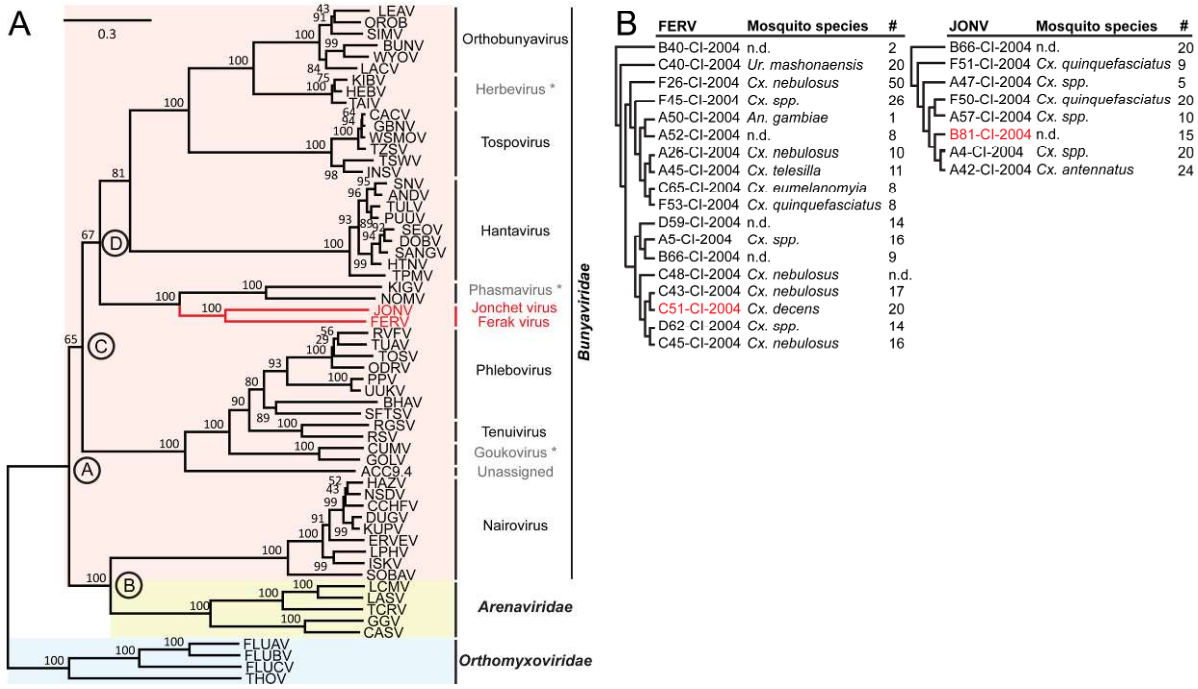


Figure IV.3 Phylogentic relationship of JONV and FERV. (A) ML analyses of polymerase proteins of bunyaviruses, arenaviruses, and orthomyxoviruses. Capital letters in circles indicate tree nodes for which ancestral hypothesis testing was performed [refer to Figure IV.5]. Bootstrap values are indicated at tree nodes. Refer to Supplement Figure IV.7 for full virus names and accession numbers. (B) Cladogram and hosts of JONV and FERV isolates.

Within this region, the two novel viruses had low degrees of amino acid identity with representatives of any bunyavirus genus or unclassified bunyaviruses [Supplement Figure IV.2C]. Both viruses' L proteins contained putative endonuclease domains and conserved palm motives [Supplement Figure IV.3].

ML phylogeny using orthomyxoviruses as an outgroup yielded a stable and reconciled topology of the bunyavirus family tree (Ballinger *et al.*, 2014; Plyusnin *et al.*, 2012) [Figure IV.3A; refer to Supplement Figure IV.4 for the alignment]. As proposed earlier, the family *Arenaviridae* was closely related to bunyaviruses, and in particular to the genus *Nairovirus* (Vieth *et al.*, 2004). The genus *Nairovirus* was placed in basal relationship to all other bunyaviruses. The next bifurcation separated the genus *Phlebovirus*, the clade containing GOLV (tentatively referred to as Goukovirus), and tenuiviruses from all other bunyaviruses. In sister relation to the clade containing the three deep-rooted lineages of JONV, FERV, and phasmaviruses was the common ancestor of the genera *Hantavirus*, *Tospovirus*, and *Orthobunyavirus* as well as the insect-restricted novel clade defined by HEBV [tentatively named Herbevirus (Marklewitz *et al.*, 2013)].

The M segments of JONV and FERV comprised ORFs of 1,730 and 1,262 aa in cRNA sense that are predicted to encode 193- and 144-kDa GPC proteins, respectively [Figure IV.2 A and D]. No similarities to any known gene were identified on the nucleotide level. The translated JONV M ORF showed low similarities to the glycoprotein gene of yellow head virus ($_{635}$ISYF-SRQV$_{1541}$; 23% identity), a crustacean infecting positive strand RNA virus in the family *Roniviridae*, order *Nidovirales*. There was also similarity to a shorter region to the GPC protein of hantaviruses ($_{965}$IDSM-LNRV$_{1168}$; 22% identity). No similarities to any viral or cellular protein were identified for the FERV GPC. Putative transmembrane domains, N-linked glycosylation sites, and signal peptide cleavage sites were predicted [Figure IV.2 A and D]. The FERV M segment encodes a second short ORF in a -1 reading frame that overlaps the N terminus of the GPC ORF by 32 nt [Figure IV.2D]. For the predicted 12-kDa protein, no similarities to any viral or cellular proteins were identified.

The JONV and FERV S segments comprised ORFs of 372 and 306 aa in cRNA sense that putatively encode 42- and 33-kDa proteins, respectively [Figure IV.2 A and D]. These correspond on genome position to nucleoprotein ORFs in other bunyaviruses. No primary sequence similarities between JONV and FERV nucleoprotein ORFs and those of other bunyaviruses were identified. Notably, both novel viruses did not encode ORFs for a putative NSs protein, according to coding strategies used for NSs proteins in other bunyaviruses, such as an NSs ORF fully overlapping the N ORF in orthobunyaviruses or an NSs ORF encoded in ambisense in phlebovirus and tospovirus S segments (Plyusnin *et al.*, 2012). Both viruses comprised ORFs of 119 and 107 aa in a -1 reading frame in cRNA sense upstream of the putative N ORF that were predicted to encode proteins of 13 and 12 kDa, respectively. No conserved protein motifs could be identified for the unassigned ORFs. The mutation frequency in these small ORFs compared with N, GPC, or RdRp ORFs was similar, suggesting the small ORFs are expressed [Supplement Figure IV.2D; also see following].

IV.3.3 Genome Replication, Transcription, and Expression

Bunyavirus replication involves the transcription of negative-sense viral RNA into cRNA acting as the replicative intermediate, with concomitant transcription of a shorter form of coding-sense RNA acting as the mRNA for protein expression (Plyusnin *et al.*, 2012). Genomic-length and slightly shorter virus-specific RNAs for the S segment were detected in cells infected with JONV by Northern blot analysis [Figure IV.2B]. For the JONV M and L segments, as well as for all FERV genome segments, only one RNA species was detected by Northern blot analysis [Figure IV.2 B and E]. To discriminate vRNA and mRNA, the amounts of positive- and negative-sense viral RNA were quantified by hot-started strain-specific reverse transcription and real-time PCR [Supplement Figure IV.5]. Genome RNA exceeded mRNA initially. Over time, mRNA exceeded genome RNA until both species reached similar levels at 24 hpi, suggesting that both RNA polarities existed in cells and mRNAs were not discriminable in size from vRNAs by Northern blot analysis. Northern blot analysis with S segment-specific probes upstream of the putative NSs ORF yielded no differences in sizes of detected RNA, suggesting no additional mRNA is transcribed for the putative NSs ORFs [Figure IV.2 B and E]. Bunyavirus mRNAs typically contain 5'-nonvirally templated elements obtained from host cell mRNAs by a cap-snatching mechanism (Bishop *et al.*, 1983; Simons & Pettersson, 1991). For confirmation, cell lysates were subjected to 5'-RACE with subsequent cloning and analysis of five cDNA clones with appropriate insert sizes per genome segment per virus. Between six and 23 nonvirally templated residues were detected at 5'-ends [Supplement Figure IV.6], indicating cap-snatching as in other bunyaviruses.

Expressed structural proteins and glycoprotein cleavage sites of JONV and FERV were assessed by SDS/PAGE, followed by limited tryptic digestion and MALDI-TOF, liquid chromatography mass spectrometry analysis, and Edman degradation from gradient purified viral particles. Six proteins of about 250, 200, 100, 80, 50, and 15 kDa were identified for JONV and mapped to the RdRp, GPC, Gn, Gc, and N genes, as well as to the unassigned ORF upstream of the N ORF, respectively [Figure IV.2C]. According to the nomenclature used for bunyaviruses, the protein was named NSs. The N-terminal sequence of the Gn protein was identified to start at $_{349}$DTGA, suggesting that the region upstream of the Gn protein codes for a putative NSm protein of 39 kDa. For FERV, three prominent bands corresponding to 80, 75, and 40 kDa were observed and confirmed to represent the Gn, Gc, and N proteins [Figure IV.2F]. The observed molecular masses for Gn and Gc were higher than the predicted ones and were compatible with glycosylation. Whereas the weight of JONV glycoproteins was reduced by treatment with peptide-N-glycosidase F, no such evidence for N-linked glycosylation was found for FERV [Supplement Figure IV.7].

IV.3.4 In Vitro Host Range and Sensitivity to Temperature.

To determine growth kinetics of the novel viruses, end-point infectious titers were first determined by parallel serial dilution experiments in C6/36 cell cultures, followed by microscopic inspection for CPE 7 dpi. Peak viral titers were 1.78 × 108 and 1.33 × 108 tissue

culture infectious dose 50/ml for JONV and FERV, respectively. To determine growth kinetics, C6/36 cell cultures were inoculated with virus at defined MOIs, and supernatants were sampled daily. Determination of virus RNA concentrations in supernatants by real-time RT-PCR indicated that FERV took 1–2 d longer than JONV to reach peak titers, in particular at lower MOI [Supplement Figure IV.1 C and D]. Peak RNA concentrations in absolute quantitative RT-PCR ranged around 10^{10} copies/mL for both viruses. Similar growth characteristics were observed for JONV in U4.4 cells, an *Aedes albopictus* cell line that is competent for the RNAi pathway [Supplement Figure IV.1C]. Growth of FERV was delayed and only detected after 48 hpi in U4.4 cells, reaching RNA concentrations similar to JONV thereafter [Supplement Figure IV.1D]. In contrast to C6/36 cells, both viruses did not induce cytopathic effects in U4.4 cells. The cell line C7/10 derived from *Aedes albopictus* larvae also supported replication of both viruses [Supplement Figure IV.1 E and F].

To determine the potential to grow in vertebrate cells, primate, rodent, bat, goat, and frog cells were inoculated with both viruses and cultivated for 4 wk. No growth, as evident by real-time RTPCR, was seen in any of the vertebrate cell cultures with any virus [Supplement Figure IV.1 E and F]. Temperature permissiveness was used as an additional criterion for potential vertebrate tropism. To compare JONV's and FERV's permissiveness at vertebrate body temperatures, arboviruses were selected from the two pathogenic bunyavirus genera *Phlebovirus* and *Orthobunyavirus*, as well as their insectrestricted sister taxa, Goukovirus and Herbevirus, and cultured under temperature gradients. This involved RVFV as an arbovirus representative for the genus *Phlebovirus* and GOLV as a bona fide insect-restricted virus of the Goukovirus clade (Marklewitz *et al.*, 2011). For the genus *Orthobunyavirus*, the arbovirus La Crosse virus as well as the prototypic insect-restricted virus HEBV were chosen (Marklewitz *et al.*, 2013). Both tested arboviruses replicated efficiently across the whole temperature range of 29–34 °C [Figure IV.4]. All four bona fide insect-restricted viruses were impaired in replication above 32 °C and completely blocked above 33 °C. Some viruses were impaired already, starting from 29 °C. FERV and JONV were completely blocked above 30 and 32 °C, respectively. To exclude that JONV's and FERV's inability to replicate in vertebrate cells might only be determined by temperature conditions, JONV, FERV, GOLV, and HEBV were infected in Vero cells at MOI of 10 and incubated at the permissive temperature of 30 °C. No viral replication was evident by real-time RT-PCR for any of the viruses, whereas RVFV and LACV replicated as previously observed.

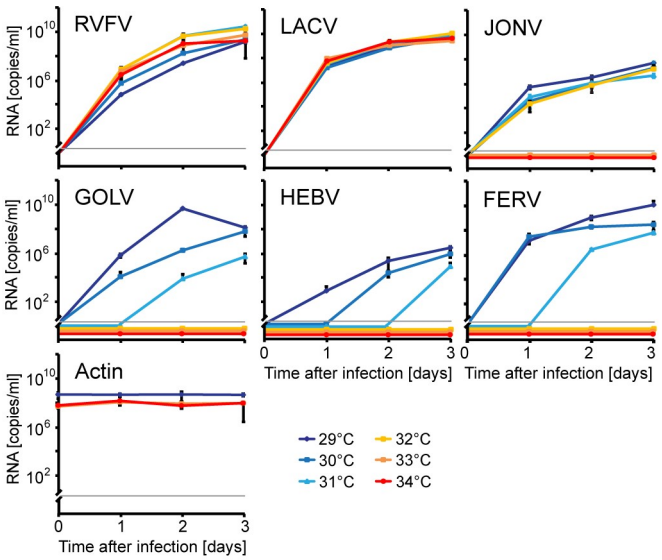


Figure IV.4 Temperature sensitivity of bunyaviruses. C6/36 cells were infected with the indicated viruses, and cells were incubated at temperatures from 29 to 34 °C. Viral genome copy numbers were measured by real-time RT-PCR. Actin copy numbers in mock-infected cells were determined for 29, 33, and 34 °C. Limit of detection is indicated by a gray line.

IV.3.5 Ancestral Reconstruction

To determine whether the novel viruses might have evolved independently to be restricted to arthropods or inherited their arthropod restriction as a property from common ancestors, phylogeny-based reconstruction of ancestral traits was attempted. We used a parsimony-based algorithm that reconstructs ancestral traits at all internal tree nodes by calculating the minimum number of trait changes along the tree that is necessary to explain the present state of host associations at tree tips (Maddison & Maddison, 2014). The model used two binary host traits that identify whether a virus has an arthropod host (yes/no) or vertebrate host (yes/no). Using this approach, an arthropod host was reconstructed at the bunyavirus root in 100% of 1,000 bootstrap tree replicates used for the analysis [Figure IV.5A]. Association with a vertebrate host could not be deduced [Figure IV.5B]. However, these analyses did not account for the uncertainty about associations with vertebrate hosts for a number of novel viral taxa included in the tree (JONV, FERV, herbeviruses, goukoviruses, phasmaviruses). Moreover, parsimony-based models cannot take branch lengths into account, which vary considerably in the given tree. To incorporate branch length information and optionally include new knowledge about host associations based on cell culture studies, we conducted probabilistic hypotheses testing in a maximum likelihood framework (Drexler *et al.*, 2012; Pagel *et al.*, 2004). This approach determines the most likely trait change matrix along the bunyavirus phylogeny as well as the loss of likelihood that occurs when restricting traits at given tree nodes (Drexler *et al.*, 2012; Pagel *et al.*, 2004). Host properties were ascribed to tree taxa in the form of combined binary traits as summarized in Supplement Table IV.1. In one dataset version termed the uninformed dataset, known host traits were ascribed to all bunyaviruses except the novel viruses JONV, FERV, GOLV, HEBV, and KIBV, for which host traits were left open. In an alternative,

informed dataset, the host trait information obtained from cell culture infection experiments in the present study was added. Using the program Bayestraits (Pagel *et al.*, 2004), a hypothesis-free reconstruction run was performed for reference, recording the median likelihood of trait change reconstructions over 1,000 bootstrap tree replicates. Fossil host assumptions were then defined at deep tree nodes, restricting the optimization space for the ML algorithm. This analysis was conducted on three different alternative tree topologies, as shown in Supplement Figure IV.8. Analysis of the uninformed dataset failed to reject any host hypotheses on a high significant level [Figure IV.5C and Supplement Figure IV.8]. Only the analysis of the informed dataset convincingly rejected vertebrate hosts and dual hosts at all deep nodes, including the root. This was unanimously the case for all alternative tree topologies, including a topology that assumed arenaviruses to belong to the bunyavirus family, as proposed earlier (Vieth *et al.*, 2004) [Supplement Figure IV.8]. Vertebrate hosts at all analyzed deep nodes were 158–794-fold less likely than arthropod hosts. Dual hosts were 63–398-fold less likely. Hypotheses of exclusive arthropod association at all deep nodes including the root left the overall likelihood unaffected [Figure IV.5C and Supplement Figure IV.8]. Discussion In this study we characterized prototype strains and numerous additional viral isolates representing two novel groups of viruses within the family *Bunyaviridae*. The type viruses, JONV and FERV, branch from an old common ancestor in sister relationship to unclassified phasmavirid sequences (Ballinger *et al.*, 2014). The novel viruses and Phasmaviruses branch deep in the bunyavirus family tree and in sister relationship to a viral superclade comprising the three established genera *Orthobunyavirus*, *Tospovirus*, and *Hantavirus*, as well as the novel unclassified clade of Herbeviruses (Marklewitz *et al.*, 2013). The lengths of the phylogenetic stem lineages of JONV and FERV, respectively, are similar to stem lineage lengths for other accepted genera including *Orthobunyavirus*, *Tospovirus*, *Phlebovirus*, and *Nairovirus*. The deep phylogenetic separation between JONV and FERV and the low level of amino acid identity suggest these viruses define two independent novel genera. KIGV and NOMV might as well define two additional independent genera, pending further study of live viral isolates (Ballinger *et al.*, 2014).

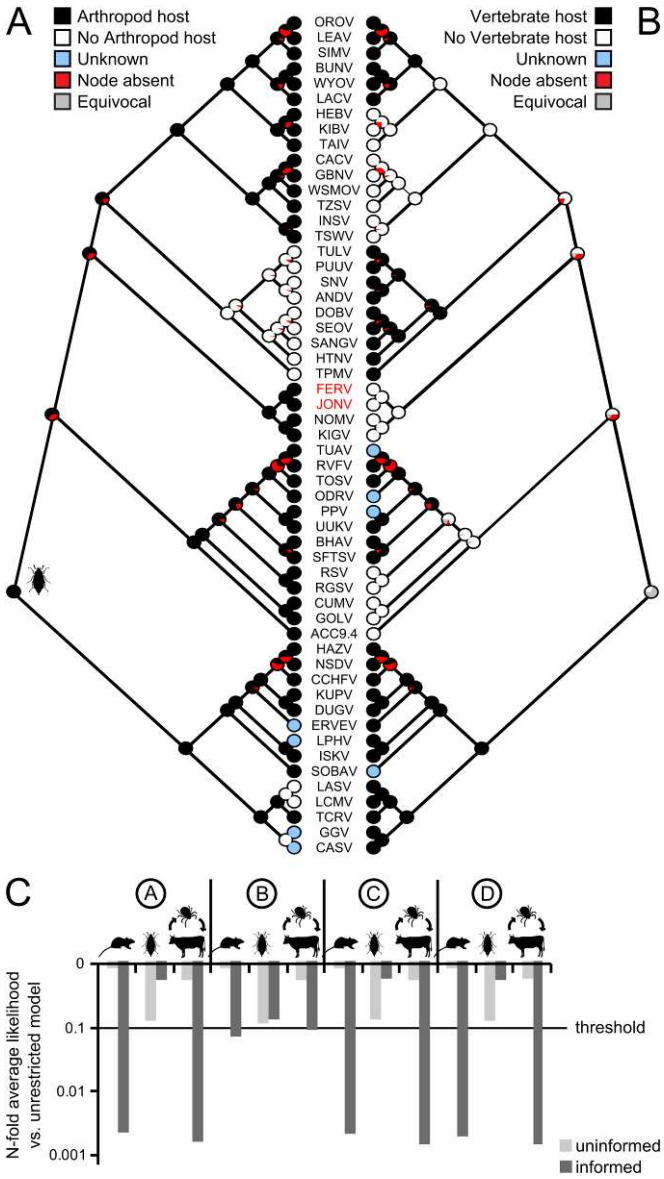


Figure IV.5 Ancestral reconstruction of bunyavirus hosts. (A and B) Parsimonybased ancestral reconstruction of arthropod (A) and vertebrate (B) host associations. "Node absent" refers to the fraction of 1,000 bootstrap tree replicates in which the node of interest was not supported (i.e., loss of monophyly of dependent clade). (C) Hypotheses testing based on ML inferences of trait change models. Hypothesized (fossilized) ancestral host assumptions at deep tree nodes A–D [refer to Figure IV.3] are symbolized by vertebrate and arthropod silhouettes. Bars represent the resulting loss of likelihood of trait change models conferred by fossilization (averaged results over 1,000 bootstrap tree replicates). The significance threshold was 10-fold loss of likelihood.

Unfortunately, the classification of bunyavirus genera does not obey any strictly defined criteria and would thus, in addition to phylogenetic analyses, have to rely on genetic, structural, or functional features that typically vary between and are conserved within accepted genera. As bunyaviruses are not known to show morphological heterogeneities within genera, the striking morphological differences between JONV and FERV could be considered as evidence for different genera. A genetic auxiliary criterion is the composition of genome segment termini. Seven nucleotides (AGUAGUA) are conserved between JONV and FERV, which is the same number of nucleotides conserved between the genus

Orthobunyavirus and its insect-restricted sister taxon Herbevirus. It is remarkable that eight additional nucleotides are shared between JONV L and S segments, but not the M segment, suggesting the M segment might have been acquired from an unknown source by reassortment after the separation of the FERV-specific stem lineage. However, reassortment within bunyavirus genera has not been investigated sufficiently to use it as a formal criterion for taxonomic classification.

Further criteria that are different between but shared within bunyavirus genera include the existence and coding strategy for noncoding elements as well as overall genome segment length variation. Another important difference exists in growth kinetics, particularly in RNAi-competent insect cells. Whereas the presence of an NSs ORF upstream of the N ORF is a unique feature of JONV and FERV, it represents a commonality rather than a contrast between the two taxa. NS proteins are typically involved in viral replication (Crabtree *et al.*, 2012; Szemiel *et al.*, 2012) or host cell interference (Bridgen *et al.*, 2001; Takeda *et al.*, 2002; Won *et al.*, 2007). ORFs compatible with NSs were also detected in KIGV and NOMV that share a MRCA with JONV and FERV (Ballinger *et al.*, 2014). The striking differences in CPE, virion morphology, genome organization, and sizes of expressed proteins, as well as phylogenetic separation, provide support for the definition of JONV and FERV as independent taxonomic entities at the rank of genera. If we add these viruses to our previous findings of Herbeviruses and Goukoviruses, four highly diverged clades that might form novel genera are now known in mosquitoes originating from one tropical rainforest region (Marklewitz *et al.*, 2011, 2013). These data nearly double the number of major bunyavirus taxa and suggest that more diverse bunyaviruses exist in other regions and arthropod species. Phasmavirids may not provide the only further example (Ballinger *et al.*, 2014).

We are currently lacking consensus experimental conditions to evaluate whether previously unknown pathogens discovered in hematophagus arthropods are arboviruses or arthropod-specific viruses. Classification of arthropod-specific viruses has so far relied on multiple negative in vitro and in vivo experiments, such as the absence of viral replication in cell lines derived from different vertebrate hosts (Evangelista *et al.*, 2013; Huhtamo *et al.*, 2009, 2014; Junglen *et al.*, 2009b; Kuno, 2007; Marklewitz *et al.*, 2011, 2013; Nasar *et al.*, 2012) or replication studies in intracerebrally inoculated newborn mice (Attoui *et al.*, 2005; Auguste *et al.*, 2014; Evangelista *et al.*, 2013; Huhtamo *et al.*, 2014). In the case of flaviviruses, in vitro replication studies of mosquitoborne, tick-borne, and insect-specific viruses, as well as vertebrate flaviviruses of the “no known-vector” group, were in agreement with the natural host range and phylogenetic grouping of these viruses, providing evidence that in vitro replication studies can be useful for host range evaluation (Kuno, 2007). Additional in vivo infection experiments, particularly in neonatal mice, have been used, but to our knowledge they yielded no examples of arboviruses replicating in neonatal mice if they cannot replicate in vertebrate cell culture. To provide an ecologically more relevant scenario and avoid animal experiments, we chose temperature gradient kinetics in cell culture. Arboviruses have to be capable of replicating at high temperatures according to the body temperature of mammals and birds (36.5–42 °C). In contrast, arthropod-restricted

viruses should be adapted to ambient temperatures, which range around 28 °C in the studied tropical rainforest habitat. Temperature gradient experiments followed the rationale that departure from ambient temperature will only be tolerated if the virus has adapted the capability to replicate at higher temperature by selection in dual host replication cycles (Aliota & Kramer, 2012). Prototype arbo-bunyaviruses from the genera *Phlebovirus* and *Orthobunyavirus* indeed were not affected by temperature, as expected because of their vertebrate tropism. As expected, all tentative arthropod-specific viruses were highly sensitive to temperature. Viral replication was completely blocked at temperatures above 31–33 °C, depending on the virus. In summary, these findings suggest that JONV and FERV, as well as the clades containing GOLV and HEBV, are insect-specific viruses without any vertebrate tropism. Vertical transmission (transovarial and transveneral) plays a major role for the transmission of insect-specific flaviviruses (Bolling *et al.*, 2011; Saiyasombat *et al.*, 2011). Other routes of transmission may also be important for the insect-specific bunyaviruses that have diverse insect host associations.

Our studies of ancestral trait reconstruction have taken these new data on insect-specific viruses into account. Whereas the parsimonybased approach could not provide any conclusive reconstruction of ancestral traits, particularly for the vertebrate association of ancestral bunyaviruses, the more comprehensive hypothesis testing studies led to a clear rejection of vertebrate hosts at deep bunyavirus nodes, including the common bunyavirus ancestor. In addition, a dual host tropism was rejected if informing the model with the novel experimental insight from temperature gradient cultures. In summary, these data suggest arbo-bunyaviruses have evolved from arthropod specific ancestors. Rejection of the vertebrate host hypothesis for several deep sister nodes in the bunyavirus tree implies that vertebrate or dual host tropism must have evolved several times convergently. The existence of different arbo-bunyaviruses in humans or mammalian livestock demonstrates that no barriers such as population immunity exist against convergent conquest of host. For the genus *Hantavirus*, our analysis infers that arthropod tropism has been lost in favor of vertebrate monotropism, rather than preserving vertebrate tropism from ancestral viruses. This may have happened with or without a transitory stage via dual host tropism (Yu & Tesh, 2014). It is tempting to speculate that the loss of dual or arthropod tropism must have taken place in mammals ancestral to bats and insect eaters, whose extant relatives seem to contain the largest diversity of hantaviruses (Guo *et al.*, 2013; Plyusnin & Sironen, 2014), and come in contact with arthropods intensively via diet. Similar scenarios may be applicable to explain the origin and evolution of other viral families that contain arboviruses and vertebrate viruses at the same time, such as the flavivirus family, wherein at least three extant genera (*Hepacivirus*, *Pegivirus*, *Pestivirus*) are thought to have exclusive vertebrate tropism.

IV.4 METHODS

IV.4.1 Mosquito Collection and Species Identification

Mosquitoes were trapped in forest edge habitats in Taï National Park, Côte d'Ivoire, as previously described (Junglen *et al.*, 2009a). Mosquito species were identified by morphologic criteria (Edwards FW, 1941; Gillies & de Meillon, 1968; Jupp, 1996). Female mosquitoes (n = 4,839) were divided into 432 pools (1–50 specimen per pool) according to mosquito species, sex, and sampling location (Junglen *et al.*, 2009a).

IV.4.2 Virus Isolation, Purification, and Growth

Virus isolation was performed in C6/36 (*Aedes albopictus*) cells (Igarashi, 1978) and in VeroE6/7 (*Ceropithecus aethiops*) cells, as described before (Junglen *et al.*, 2009b). Briefly, mosquito pools were homogenized and 100 µL of the clarified supernatant was used to infect the cells. The viruses in this study were three times endpoint-diluted, and virus stocks of the eighth passage of FERV (isolate C51/CI/2004) and the ninth passage of JONV (isolate B81/CI/2004) were used for further analyses. Virus titers were determined by tissue culture infectious dose 50 in C6/36 cells (Reed & Muench, 1937). For virus growth kinetics, C6/36 cells were infected in doublets at MOI of 0.1 and 0.01 (Zirkel *et al.*, 2011). Aliquots of infectious cell culture supernatant were harvested every 24 h for periods of 5 d, and viral genome copies were quantified by real-time RT-PCR [FERV-F 5'-TCAGCTAGTCAGATACCATCAATAC, FERV-R 5'-CAATGTTACTACAGTCGGCTTTTTTG, FERV-TM 5'- 6-carboxyfluorescein(FAM)-CCCAATATGCAAGATTCAGGGACAGAA-BHQ1; JONV-F 5'-TGCTTCGGAAGGAGCTCTCTA, JONV-R 5'-TGTATGGCTCAAGTGCCTCTAATC, JONV-TM 5'-6-FAM-AGGAAAAAGCAAGTCAGCACTCCTCGC-BHQ1]. The detection limit of each assay is below 10,667 viral genome copies per milliliter (projected).

IV.4.3 Cell Culture Infection Experiments

African green monkey kidney epithelial cells (VeroE6/7), baby hamster kidney fibroblasts (BHK-21), human hepatocellular carcinoma cells (HuH-7), horseshoe bat lung cells (RhiLu/1) (Hoffmann *et al.*, 2013), Aba roundleaf bat lung cells (HipaLu/24), kidney cells from goats (*Capra hircus*) (ZN-R), lung cells from Lesser white-toothed shrew (Crocsu-Lu) (Eckerle *et al.*, 2014), and cells derived from grass frog embryos (*Rana pipiens*) (ICR-2a) were infected with JONV or FERV at an MOI of 10. Cell culture supernatants were passaged in fresh cells every 7 d in a 1–10 dilution for three consecutive passages. Supernatants from passages 0, 1, and 3 were tested for virus replication by real-time RT-PCR.

To assess temperature sensitivity, C6/36 cells were infected with JONV, FERV, GOLV, HEBV, LACV, or RVFV at an MOI of 0.1 and cultured under temperature gradients from 29 to 34 °C for 3 d, respectively. VeroE6/7 cells were infected with JONV, FERV, LACV, or RVFV at an MOI of 10 and incubated at 30 °C. Mock infected cells were incubated at 29, 33, and

34 °C. RNA was extracted from cell culture supernatants 0, 24, 48, and 96 hpi. Viral genome copies and actin gene copies were quantified by real-time RT-PCR.

IV.4.4 Electron Microscopy

Virions were sedimented through a 36% (vol/vol) sucrose cushion by ultracentrifugation, resuspended in PBS, and fixed with 2% (vol/vol) paraformaldehyde (Biel & Gelderblom, 1999; Hayat MA, 2000). For ultrathin sections, infected cells were fixed with 2.5% (vol/vol) glutaraldehyde, enclosed in low-melting agar, and embedded in resin (Biel & Gelderblom, 1999; Hayat MA, 2000). Virions were analyzed by transmission electron microscopy.

IV.4.5 Genome Sequencing

Full-genome sequencing of JONV (isolate B81/CI/2004) and FERV (isolate C51/CI/2004) was done by a combination of deep sequencing via 454-pyrosequencing on a GS Junior Platform (Roche) (Zirkel *et al.*, 2011) and random-primed RT-PCR optimized for the detection of encapsidated nucleic acids (Junglen *et al.*, 2009b; Stang *et al.*, 2005). Briefly, after RNA extraction, double-stranded cDNA was synthesized with random hexamers linked to an anchor sequence (5'-GACCATCTAGCGACCTCCAC). Amplification was performed with anchor-specific oligonucleotides by PCR. The PCR product was cloned into the pCR2.1 TOPO vector (Life Technologies). Clones were analyzed by PCR and Sanger-sequenced. Sequences were assembled using Geneious v6 (Kearse *et al.*, 2012).

Fragment-specific oligonucleotides were used to close sequence gaps by PCR. The 3' and 5' genome termini were confirmed by RACE-PCR using the 5' RACE Kit (Life Technologies). Additional full-genome sequences of JONV and FERV isolates were generated by deep sequencing on an Ion Torrent PGM platform (Life Technologies) according to the manufacturer's instructions. Reads were identified by reference mapping to JONV B81/CI/2004 and FERV C51/CI/2004, respectively, and whole genomes were generated under visual inspection in Geneious (Kearse *et al.*, 2012).

IV.4.6 Virus Prevalence Screening and Sequence Generation

C6/36 cell cultures were screened for the presence of JONV and FERV with virus-specific real-time RT-PCR (Junglen *et al.*, 2009a). From all JONV- or FERVpositive mosquito pools, a genome fragment comprising the palm domain of the RdRp was amplified by nested PCR. Primers for the first PCR were JONVF1 5'-TGGATCATGGACACAAGGCCACTC and JONV-R1 5'-GCCCTCTTGGCAGTAAGCCACC, and FERV-F1 5'-AACCACAGCAATGCTATCTGGGC and FERV-R1 5'-AACCACAGCAATGCTATCTGGGC, respectively. Nested PCR was performed with JONV-F2 5'-GGAAGGGCTGCATATCAAGGG and JONV-R2 5'-CCCTGCATCCAACCAATCCTACC, or FERVF2 5'-CAGGTCATCAAGGAATACCCAGAG or FERV-R2 5'-CCAACCTGCTACTCCTCTTATGCT, respectively. The PCR products were Sanger-sequenced after purification.

IV.4.7 Genome and Phylogenetic Analyses

The genome was analyzed by comparison of the nucleotide and amino acid sequences with other sequences of the GenBank database (www.ncbi.nlm.nih.gov/Genbank), using BLASTn and BLASTx. Conserved protein motifs were identified by web-based comparison with the Pfam database (pfam.xfam.org/). Putative signal peptide cleavage sites were identified with the SignalP prediction server (www.cbs.dtu. dk/services/SignalP). Hydrophobic and potential transmembranespanning regions were predicted by transmembrane helices in proteins (TMHMM) (www.cbs.dtu.dk/services/TMHMM-2.0), and N-linked glycosylation sites were identified using the NetNGlyc 1.0 server (www.cbs.dtu.dk/services/NetNGlyc).

For phylogenetic analysis, complete RdRp protein sequences were aligned with representative sequences of other bunyaviruses, using the Expresso structural alignment algorithm on the TCoffee webserver (tcoffee.crg.cat) (Notredame *et al.*, 2000). The alignment was manually inspected, and poorly aligned columns were removed, resulting in a final alignment of 411 amino acids. For phylogenetic analyses including outgroups, complete translated ORFs of arenavirus L segments and concatenated translated ORFs of othomyxovirus segments encoding the PA and pb1 proteins were added to the stripped bunyavirus alignment and aligned using multiple sequence alignment based on fast Fourier transform (MAFFT) and the E-INS-I algorithm (Katoh *et al.*, 2002). The alignment was reduced to conserved columns consisting of 270 amino acids [Supplement Figure IV.4]. Phylogenetic analyses were performed using PhyML and the Blosum62 substitution model in Geneious v6 (Kearse *et al.*, 2012), with confidence testing based on 1,000 bootstrap iterations.

Phylogenetic analyses of JONV and FERV isolates were based on a 1,020-nt sequence alignment comprising the conserved RdRp motifs. Maximum likelihood analyses were performed using the GTR model with 1,000 replicates in Geneious v6 (Kearse *et al.*, 2012).

IV.4.8 Ancestral State Reconstruction

Parsimony-based ancestral state reconstruction was done in Mesquite, using the ancestral state reconstruction package (Maddison & Maddison, 2014) and the informed trait characteristics shown in Supplement Table IV.1. Maximum likelihood-based ancestral state reconstruction and hypotheses testing was done in Bayestraits (Pagel *et al.*, 2004). The trait change matrix was based on four states that were composed of two uncorrelated binary traits occurring in combination (state 1 = no insect host, no vertebrate host; state 2 = no insect host, vertebrate host; state 3 = insect host, no vertebrate host; state 4 = dual hosts) [Supplement Table IV.1]. Transition likelihoods between all four states were left independent. For hypotheses testing, ancestral host assumptions were fixed at tree nodes of interest, and maximum likelihood values for trait change matrices achieved after 1,000 optimization attempts were recorded across 1,000 bootstrap replicates of ML trees. The relative likelihoods between models using a fixed host assumption and the null model (no fixed assumptions) were expressed as “loss of likelihood” against the null model (there was

always a loss). This approach corresponds to a likelihood ratio test, which for the case of nested models is likelihood ratio = 2 [log-likelihood (better fitting model) − log-likelihood (worse fitting model)]. With likelihood ratio > 2 conventionally being considered significant, a relative likelihood of 1 log (corresponding to a 10-fold loss of likelihood) was considered as the threshold of significance. All analyses were replicated on 1,000 bootstrap versions of the ML trees shown in Supplement Figure IV.8 A–C.

IV.4.9 mRNA Analyses

C6/36 cells were infected with JONV and FERV at an MOI of 0.1 and harvested 24 hpi. mRNA was extracted using the RNeasy total RNA Extraction Kit (Qiagen) and analyzed by Northern blotting, as described previously (Marklewitz *et al.*, 2013; Zirkel *et al.*, 2011). Specific DIGlabeled PCR probes were generated using the primers JONV-L-F 5′-GGAAGGGCTGCATATCAAGGG, JONV-L-R 5′-CATTTTGCCTACATTGTCAGACTCAG, JONV-M-F 5′-GAGGAAGATGTAGTCAGCGAGGGAGG, JONV-M-R 5′-ACTTCAACTCCAGCAACGTGTTCG, JONV-S1-F 5′-TGCGTACAGTTGCCTTCCGG, JONF-S1-R 5′-ACCTCGCAAGTATCAGCTTACGC, JONV-S2-F 5′-CTGTTTTGGCTATGTTACCGCAGGC, JONVS2- R 5′-GTCTATTTGGCGTTGGATTTCAGCAAG, FERV-L-F 5′-TGCTAGAAGAGGCAGATATGTTGTGGG, FERV-L-R 5′-TCCCTGGTTCACCTTCAATACGG, FERV-M-F 5′-AGTAAACCTTGATTTCACCATGTCTGCTC, FERV-M-R 5′-GCAGTTTGTCAATGTTGTTAAAGCTTG, FERV-S1-F 5′-CGCATTACGTGTGATTCGTACTCG, FERV-S1-R 5′-AATTGTCAGACCTTGGTAATTAGCCTCC, FERV-S2-F 5′-CAGCTTCAGGCAGATCACTGTGC, and FERV-S2-R 5′-AGTGCCTTGATGTTGCTGTCTGTC. To discriminate between vRNA and mRNA, JONV-infected cells were harvested 0, 3, 6, 15, and 24 hpi, and RNA was extracted. Hotstarted strain-specific cDNA was synthesized using either a forward primer (5′-GAACACGTTGCTGGAGTTG) or a reverse primer (5′-TACCCACAGTCCTTGCTTGTTC). Amounts of positiveand negative-sense viral RNA were quantified by real-time PCR using the primers JONV-F-M 5′-TGGGTGAAGCTAGGGTAGAAGTAGA and JONV-R-M 5′-GTCAGACCATCCAGTGTAAAAACCT and the probe JONV-TM-M 5′-6-FAM-AGCCACTTGGCAACTCATACACGGTTCA-BHQ2.

IV.4.10 Protein Analyses

Viral proteins were analyzed as previously described (Zirkel *et al.*, 2013). Briefly, viral particles were purified by saccharose gradient ultracentrifugation. Viral proteins were either lysed directly or deglycosylated using peptide-N-glycosidase F (New England BioLabs) and subsequently lysed in 4xNuPage LDS Sample Buffer (Life Technologies) at 70 °C for 10 min. Proteins were separated by SDS/PAGE on a NuPAGE Novex 4–12% (vol/vol) Bis Tris gel with NuPAGE MES SDS Running Buffer (Life Technologies) and visualized by Coomassie blue (R-250) staining. Proteins were analyzed by limited tryptic digestion and mass spectrometry using MALDI-TOF and LC-MS. N-terminal amino acid sequencing was performed by Edman degradation, as previously described (Zirkel *et al.*, 2013).

IV.4.11 Nucleotide Sequence Accession Numbers

Complete genome sequences of JONV and FERV viruses were assigned GenBank accession numbers KP710232, KP710238-KP710245 and KP710246, KP710262-KP710269, respectively. Further sequence fragments from JONV and FERV strains were assigned to GenBank accession numbers KP710233–KP710237 and KP710247–KP710261, respectively.

5. GENERAL DISCUSSION

This thesis describes the discovery and characterization of the first insect-specific viruses of the family *Bunyaviridae*. The majority of bunyaviruses are pathogenic to vertebrates and are transmitted by blood-feeding arthropods (Schmaljohn & Nichol, 2007). This dual host tropism (arthropods and vertebrates) is a classical feature of the non-taxonomic group of arboviruses. Since the establishment of the family *Bunyaviridae* no taxon that groups outside the five established genera has been described (King *et al.*, 2012). The six novel viruses characterized in this thesis are the first that do not group within these five genera and define four distinct novel deep rooting lineages. Infection experiments with these viruses revealed that they have an atypical host tropism compared to known bunyaviruses (Marklewitz *et al.*, 2011, 2013, 2015). The novel viruses are able to infect insects but cannot infect vertebrates. All bunyaviruses previously isolated from blood-feeding arthropods are able to infect vertebrates. The host restriction of the novel viruses to insects was confirmed by a newly established method that can help to determine the host range of novel viruses isolated from blood-feeding insects (Marklewitz *et al.*, 2015). Replication of insect-specific viruses was inhibited at temperatures above 31°C and thus, at vertebrate typical temperatures (Marklewitz *et al.*, 2015). In contrast, arboviruses were not impaired by higher temperatures.

The origin and evolution of bunyaviruses has been unknown until recently. Phylogenetic analysis combined with reconstruction of ancestral host associations suggest an arthropod host as the ancestor at the root of the family *Bunyaviridae* (Marklewitz *et al.*, 2015). These data strongly support the hypothesis that the vertebrate-infecting arboviruses evolved from viruses with a monotropism for arthropods.

Six distant novel virus species were isolated from tropical mosquitoes sampled in Ghana, Ivory Coast and Uganda. The viruses were named Gouléako virus (GOLV), Herbert virus (HEBV), Taï virus (TAIV), Kibale virus (KIBV), Jonchet virus (JONV) and Ferak virus (FERV) (Marklewitz *et al.*, 2011, 2013, 2015). Several strains from each species were found. To date, the ICTV has not established formal criteria for the classification of new bunyavirus genera (King *et al.*, 2012). In phylogenetic analyses, GOLV, FERV, JONV, and the clade of HEBV, TAIV, and KIBV did not group with the established genera suggesting novel taxonomic units at the level of genera. GOLV shares a common ancestor with all members of the *Phlebovirus* genus and was proposed to define a new genus tentatively named *Goukovirus* [Figure 3] (Marklewitz *et al.*, 2011). Its minimal genome size, the lack of NSs and NSm proteins as well as the absence of serological cross reactivity with representative members of the *Phlebovirus* genus, clearly discriminate GOLV from phleboviruses. Recently, a novel bunyavirus called Cumuto virus (CUMV) was described (Auguste *et al.*, 2014). The L, M and S segments of CUMV were found to be similar to GOLV (amino acid identities are 54%, 38%, and 36%, respectively). CUMV was also shown to be insect-specific and may represent the second member of the proposed new genus *Goukovirus*. Moreover the clade of HEBV, KIBV, and TAIV was also suggested to define a new genus provisionally named *Herbevirus* (Marklewitz *et al.*, 2013). This clade forms a sister clade to the established genus *Orthobunyavirus* [Figure 3]. FERV and JONV are sister taxa that branch from a very deep

common node with the recently described phasmaviruses (Ballinger *et al.*, 2014; Junglen, 2016) [Figure 3]. Fera-, jon-, and phasmaviruses are in basal relationship to the superclade of orthobunya-, tospo-, hanta-, and herbeviruses [Figure 3]. The phylogenetic distance between FERV and JONV is similar to the genetic distance of established genera within the bunyavirus family, suggesting that these viruses define two independent novel genera namely *Feravirus* and *Jonvirus* (Marklewitz *et al.*, 2015).

Beside phylogenetic analysis, other biologic and genetic properties are used as classification criteria. The putative prototype viruses of the novel genera GOLV, FERV, HEBV, and JONV were isolated from mosquitoes and have been shown to exclusively infect insect cell lines. Members of the family *Bunyaviridae* that are capable of infecting a vertebrate host can also replicate at lower temperatures, reflecting the ambient body temperature of arthropods (Marklewitz *et al.*, 2015). This is essential due to their dual host tropism as an arbovirus. The replication of insect-specific viruses is abolished at vertebrate typical temperatures. The distinct host range of GOLV, FERV, HEBV, and JONV together with phylogenetic clustering provide strong support for the hypothesis that these viruses are prototype species of novel bunyavirus genera.

GOLV, FERV, HEBV, and JONV, the unassigned genera *Emara*- and *Tenuivirus* as well as bunyaviruses have been shown to generate viral mRNAs by a cap-snatching mechanism as an overall common feature (Marklewitz *et al.*, 2013, 2015; Nguyen *et al.*, 1997; Schmaljohn & Nichol, 2007; Walia & Falk, 2012). Phasmaviruses could not be isolated in cell culture and it remains unclear if these viruses use a cap-snatching mechanism (Ballinger *et al.*, 2014). The novel viruses described in this thesis share with bunyaviruses and the unassigned genera *Phasma*-, *Tenui*-, and *Emaravirus* the common feature of a segmented genome with reverse complementary terminal nucleotides (Ballinger *et al.*, 2014; Mielke-Ehret & Mühlbach, 2012; Schmaljohn & Nichol, 2007; Toriyama *et al.*, 1998).

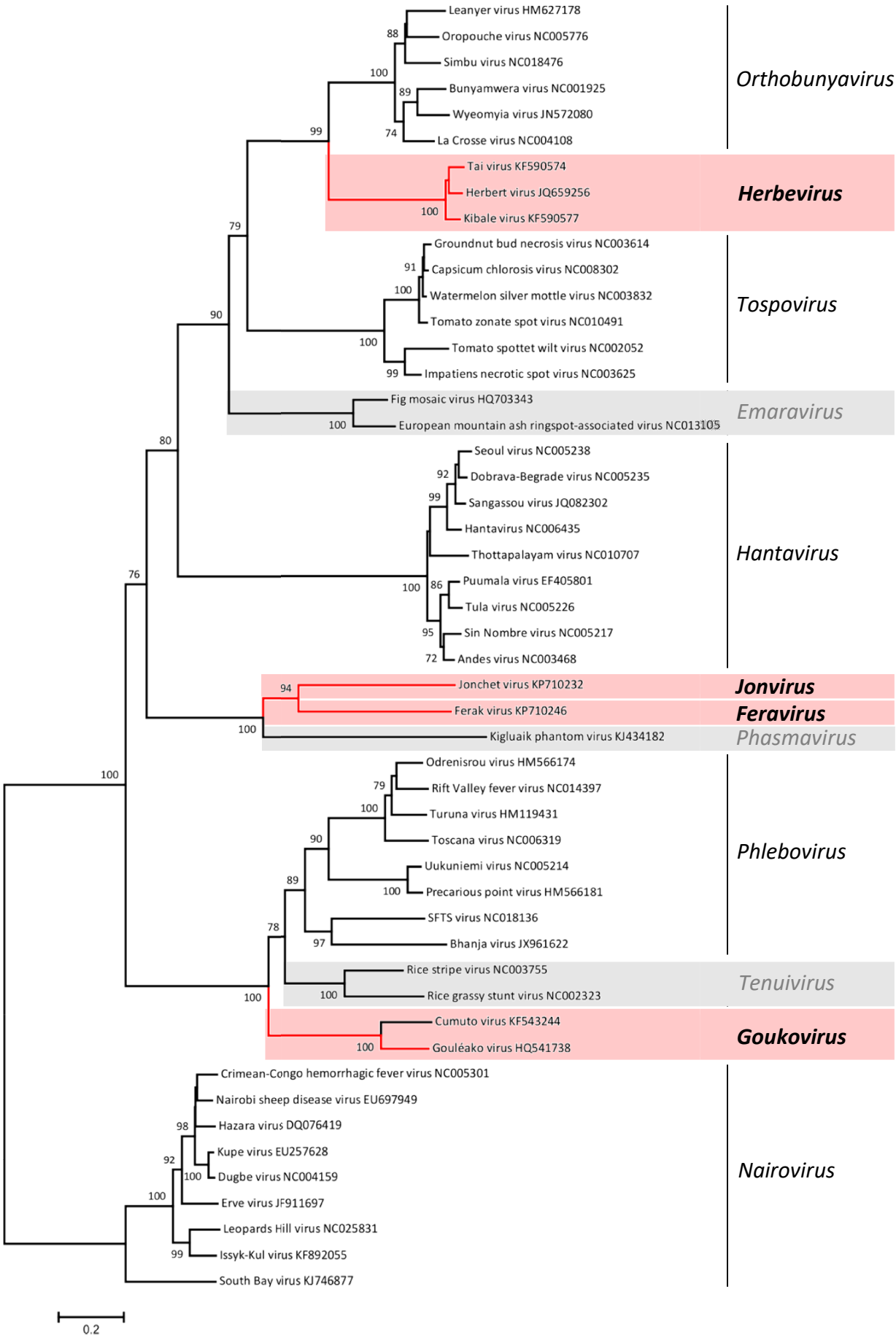


Figure 3. (Legend continued on following page)

Phylogeny of representative taxa available as live virus isolates of bunyaviruses, related unassigned, and putative novel bunyavirus genera. A stripped amino acid alignment of the conserved region of the RNA-dependent RNA-polymerase protein was created using MAFFT and the E-INS-I algorithm (Katoh *et al.*, 2002). Phylogenetic analysis was performed using PhyML implemented in Geneious 6 and Blosum62 substitution matrix and 1000 bootstrap replicates (Guindon & Gascuel, 2003; Kearse *et al.*, 2012). Grey boxes compass the unassigned genera *Emara-*, *Phasma-* and *Tenuivirus* and the proposed novel bunyavirus genera *Herbe-*, *Jon-*, *Fera-* and *Goukovirus* are highlighted with red boxes. NCBI Genbank accession numbers are indicated at each taxon.

The morphology of bunyaviruses is consistent throughout the family. Generally, bunyavirus particles are enveloped, spherical and have a diameter of 80-120 nm. A typical bunyavirus-like morphology was also found for FERV, GOLV and HEBV virions (Marklewitz *et al.*, 2011, 2013, 2015). However, the virions lacked glycoprotein projections which might explain the smaller size of some particles (60-120 nm). Furthermore, virions of the unassigned genus *Emaravirus* are also enveloped and the spherical particles are between 80-100 nm in diameter. In contrast, JONV shows a strikingly different morphology with two types of virions having either a tubular morphology with 60x600 nm or a spherical morphology of 80 nm in diameter [Figure IV.1]. Interestingly, there have been two distinct morphologies described for Influenza virus particles that are very similar to the ones of JONV (Seladi-Schulman *et al.*, 2013). The functional significance of each virion type is unclear but it has been suggested that the tubes may play a functional role in the infected host as spherical particles are dominant after serial passaging *in vitro* under laboratory conditions (Seladi-Schulman *et al.*, 2013). Further *in vivo* studies are needed to investigate whether the numbers of tubular particles of JONV are similarly dominant within the mosquito host. Tenuiviruses seem not to form viral particles and consist of RNPs only which have a thin filamentous shape (Shirako *et al.*, 2012). *Tenuivirus* RNPs differ in their length depending on the sizes of the RNAs they contain and form spiral-shaped, branched or circular structures. The morphology of the bunyavirus related unassigned genus *Phasmavirus* is unknown (Ballinger et al., 2014).

A key feature of members of the family *Bunyaviridae* is the presence of reverse complementary nucleotides at the termini of each genome segment that allow the formation of panhandle-like structures [refer to Figure 1]. These terminal nucleotides (8-11 nt) are highly conserved within bunyavirus genera but vary among these genera [Table 2]. The conserved nucleotides have been classically used to assist in the assignment of new taxa to bunyavirus genera (Plyusnin *et al.*, 2012; Schmaljohn & Nichol, 2007). FERV and JONV have seven conserved terminal nucleotides in common [Table 2]. Both viruses share six of those nucleotides with members of the genus *Orthobunyavirus*, the proposed new genus *Herbevirus* and the unassigned genus *Emaravirus*. Eight additional nucleotides are shared between the L and S segments of FERV and JONV, but not between the M segments. This observation suggest that JONV's M segment may have been aquired from an unknown source by a reassortment event. The distinct morphologies [Figure IV.1] and the low genetic identity (up to 25%) between FERV and JONV supports their classification as separate genera. GOLV shares eight terminal nucleotides of the L segment with phlebo- and tenuiviruses but only five in the M and S segment. The presence of less shared nucleotides

might be explained similarly as discussed for JONV above: by recombination or reassortment events after the evolutionary separation of gouko-, phlebo- and tenuiviruses [Table 2]. On the other hand, ten nucleotides at the terminal 5' end are identical with tenuiviruses and it underlines the genetic relationship between gouko- and tenuiviruses. Twelve nucleotides are conserved between HEBV and orthobunyaviruses, nevertheless, overall terminal nucleotides of HEBV compared to orthobunyaviruses are truncated. This supports *Herbevirus* being a separate bunyavirus genus [Table 2].

Table 2. Terminal nucleotides of genome segments. Reverse complement nucleotides per segment at 3' and 5' end in bold. Nucleotides conserved over segments among species indicated by black line. Viruses characterized in this thesis highlighted in red boxes and their highest similarity with the respective bunyavirus or unassigned genus is colored in blue and green.

Genus Prototype virus	L/1 segment	M/2 segment	S/3 segment
Hantavirus			
Hantaan hantavirus	3' **AUCAUCAU**ACGA– 5' **UAGUAGUA**GACU–	3' **AUCAUCAUCUGU**– 5' **UAGUAGUAGACA**–	3' **AUCAUCAUC**AUA– 5' **UAGUAGUAG**ACU–
Nairovirus			
Dugbe nairovirus	3' **AGAGUUUCU**UUA– 5' **UCUCAAAGA**CAU–	3' **AGAGUUUCU**AUC– 5' **UCUCAAAGA**CAU–	3' **AGAGUUUCU**CUA– 5' **UCUCAAAGA**CAA–
Orthobunyavirus			
Bunyamwera orthobunyavirus	3' **AGUAGUGU**ACUC– 5' **UCAUCACA**CGAG–	3' **AGUAGUGU**ACUA– 5' **UCAUCACA**CGAU–	3' **AGUAGUGU**ACUC– 5' **UCAUCACA**CACG–
Phlebovirus			
Rift Valley fever phlebovirus	3' **ACACAAAG**GCGC– 5' **UGUGUUUC**UGGC–	3' **ACACAAAGAC**GG– 5' **UGUGUUUCUG**GC–	3' **ACACAAAG**ACCC– 5' **UGUGUUUC**GAGG–
Tospovirus			
Tomato spotted wilt tospovirus	3' **AGAGCAAUCAGG**– 5' **UCUCGUUAGUCC**–	3' **AGAGCAAUCAGU**– 5' **UCUCGUUAGUCA**–	3' **AGAGCAAU**UGUG– 5' **UCUCGUUA**GCAC–
Unassigned			
Emaravirus†			
European mountain ash ringspot-associated virus*	3' **AGUAGUG**AACUC– 5' **UCAUCAC**AAGAG–	3' **AGUAGUG**AACUC– 5' **UCAUCAC**AAGAG-	3' **AGUAGUG**AACUC– 5' **UCAUCAC**AAGAG–
Phasmavirus#			
Kigluaik virus	3' **AGCAGCA**CGCAA– 5' **UCGUCGU**ACGAU–	3' **AGCAGC**ACGCAA– 5' **GUCGUC**GUACGA–	3' **AGCAGCA**CGCAU– 5' **UCGUCGU**ACGAG–
Tenuivirus†			
Rice grassy stunt virus*	3' **ACACAAAGUC**CU– 5' **UGUGUUUCAG**AC–	3' **ACACAAAGUC**CU– 5' **UGUGUUUCAG**AC–	3' **ACACAAAGUC**CU– 5' **UGUGUUUCAG**AC–
Feravirus#	3' **AGUAGUAAACAC**– 5' **UCAUCAUUUGUG**–	3' **AGUAGUAAACAG**– 5' **UCAUCAUUUGUC**–	3' **AGUAGUAAACAG**– 5' **UCAUCAUUUGUC**–
Ferak virus			
Goukovirus#	3' **ACACAAAG**ACAC– 5' **UGUGUUUC**AGGU–	3' **ACACA**GUGACCC– 5' **UGUGU**UUCAGGG–	3' **ACACA**GUGACCU– 5' **UGUGU**UUCAGGG–
Gouléako virus			
Herbevirus#	3' **AGUAGUGUGCU**U– 5' **UCAUCACACGA**G–	3' **AGUAGUG**UGCAU– 5' **UCAUCAC**GUGUA–	3' **AGUAGUG**UGCAU– 5' **UCAUCAC**GUGUA–
Herbert-, Kibale-, Taï virus			
Jonvirus#	3' **AGUAGUAUACCG**– 5' **UCAUCAUAUGGC**–	3' **AGUAGUA**CACCG– 5' **UCAUCAU**AUGGC–	3' **AGUAGUAUAC**CG– 5' **UCAUCAUAU**GAC–
Jonchet virus			

#proposed genera †Genome contains more than three segments *non-prototype species

The coding and expression strategies of nonstructural proteins (NSs and NSm) can be used as additional criteria to assign novel taxa to a bunyavirus genus. These proteins are typically responsible for counteracting the host immune system (Bridgen *et al.*, 2001; Takeda *et al.*, 2002; Won *et al.*, 2007) or are involved in viral replication (Crabtree *et al.*, 2012; Szemiel *et al.*, 2012). Orthobunyaviruses encode an NSs protein within the N ORF in the same coding direction (Schmaljohn & Nichol, 2007). In contrast, phlebo- and tospoviruses encode their NSs proteins in ambisense orientation in non-overlapping ORFs separated from the N ORF by a hairpin structure (Giorgi *et al.*, 1991; Simons *et al.*, 1990). The sizes of typical NSs proteins vary between 29 and 52 kDa. No putative NSs protein gene was identified in the genome of the insect-specific GOLV encoded in a similar strategy, as seen in other bunyaviruses. However, an ORF present in sense orientation within the N ORF of GOLV was identified (Marklewitz *et al.*, 2011). Whether this ORF is expressed and may represent an NSs protein, needs further investigation. The S segments of the insect-specific viruses HEBV, KIBV and TAIV show no evidence for ORFs encoding for any functional NSs. Furthermore, the S segment of FERV and JONV contain NSs ORFs in the same orientation and partially overlapping with the N ORF. The expression of this ORF in JONV was documented by SDS/PAGE and confirmed by mass spectrometry (Marklewitz *et al.*, 2015). Phasmaviruses seem to encode a NSs protein in a similar coding strategy as found for FERV and JONV [Figure 4] (Ballinger *et al.*, 2014). A recently described orthobunyavirus named Brazoran virus (BRZV) contains an unusually large S segment that encodes an NSs protein in a similar manner as observed in FERV, JONV and phasmaviruses (Lanciotti *et al.*, 2013). The NSs ORF of BRZV is preceding the N ORF but the overlapping part is significantly shorter than in JONV, FERV and phasmaviruses.

The insect-specific gouko- and herbeviruses do not encoding NSs or NSm proteins within their genomes. Gouko- and herbeviruses branch from deep nodes in the bunyavirus tree and share a common ancestor with phlebo- and orthobunyaviruses, respectively. The latter encode NSs and NSm proteins suggesting that NSs and NSm proteins were acquired convergently in both genera. A putative independent acquisition is confirmed by different coding strategies in phlebo- and orthobunyaviruses. It is likely that these proteins have evolved with the ability to infect vertebrate hosts (Billecocq *et al.*, 2004). In contrast to gouko- and herbeviruses, FERV and JONV which are also insect-specific viruses, do encode a NSs protein. The protein may play a role in the modulation of the immune response of mosquitoes. It has been shown that the NSs protein of tospoviruses acts as an RNAi inhibitor in plant hosts (Hedil *et al.*, 2015; Schnettler *et al.*, 2010; Takeda *et al.*, 2002). As tospoviruses have a dual host tropism infecting plants as well as their arthropod vector (thrips) (Wijkamp *et al.*, 1993), they encounter an RNAi defense mechanisms in both hosts. Whether the NSs proteins of FERV and JONV have an RNAi antagonistic function needs to be addressed in future studies.

Emara- and tenuiviruses also encode proteins with functions homologous to the bunyavirus NSs proteins. The tenuivirus Rice hoja blanca virus (RHBV) infects rice plants and is transmitted by the insect vector, *Sogatodes oryzicola* in which it is also transovarially transmitted (Ramirez *et al.*, 1992; Zeigler & Morales, 1990). One of the four genome

segments of RHBV (RNA3) encodes a 27 kDa protein namely the nonstructural protein 3 (NS3). This protein has been identified to be responsible for RNAi suppression in plants and in insect cells, similar to the NS3 protein of tenuiviruses and the NSs protein of tospoviruses (Bucher *et al.*, 2003; Hemmes *et al.*, 2007). Furthermore, the NSs protein of tospoviruses and the NS3 protein of tenuiviruses are encoded in a similar ambisense coding strategy on the third-largest segments [Figure 4], underlining the genetic relationship between tospo- and tenuiviruses (Bucher *et al.*, 2003). To date, RNA silencing suppression activity was not detected for any of the emaraviruses (Lu *et al.*, 2015). However, due to the limited diversity discovered so far, the possible existence of a homologous protein in emaraviruses cannot be ruled out.

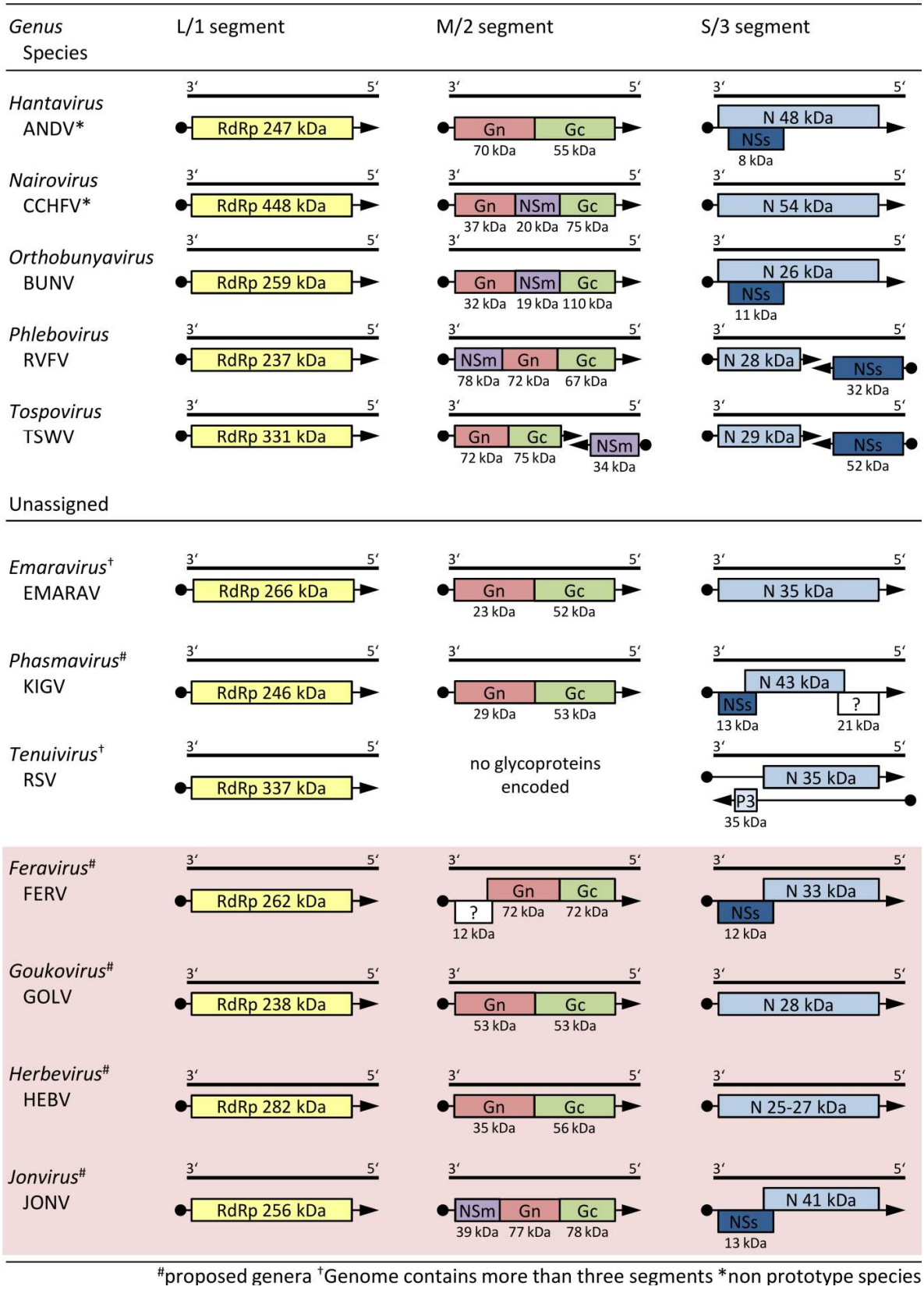


Figure 4. Protein coding strategies among bunyaviruses and related unassigned genera. Colored boxes indicate the positions and designations of the ORF translation products: RdRp protein (yellow), the glycoproteins Gn (red) and Gc (green), the nucleocapsid protein N (blue) and nonstructural proteins NSs (dark blue), NSm (violett) and P3 (light blue). Protein sizes are refering to the species indicated: *Andes hantavirus* (ANDV), *Crimean-Congo hemorrhagic fever nairovirus* (CCHFV), *Bunyamwera orthobunyavirus* (BUNV), *Rift Valley fever phlebovirus* (RVFV), *Tomato spotted wilt tospovirus* (TSWV), European mountain ash ringspot-associated virus (EMARAV), Kigluaik phantom virus (KIGV), Rice stripe virus (RSV), Ferak virus (FERV), Gouléako virus (GOLV), Herbert virus (HEBV) and Jonchet virus (JONV).

The genome organization and coding strategies for structural, as well as nonstructural proteins, may vary within a family. For example, genera of the family *Secoviridae* have segmented- and monopartite genomes present (Sanfaçon *et al.*, 2012): The Tomato torrado virus contains a two-segmented genome that encodes an N protein on segment RNA2 at the C-terminus of a precursor protein. This is in contrast to the monopartite Rice Tungro spherical virus which encodes the N protein at the N-terminus of its single polyprotein ORF (Sanfaçon *et al.*, 2012). Further examples of different coding strategies can be found in the family *Dicistroviridae* (Chen *et al.*, 2012). As a consequence, the number of genome segments within a virus family may vary. The unassigned genera *Emaravirus* and *Tenuivirus* contain more than three genome segments but the phylogenetic relationship of their RdRp proteins suggest that the viruses are members of the family *Bunyaviridae*.

Six conserved motifs, premotif A to motif E, in the palm domain of the bunyavirus RdRp proteins were described previously and were used to analyse the phylogenetic relationship of the family (Poch *et al.*, 1990). However, evidence from the genetic information of the four novel prototype insect-specific virus species suggests that the lengths of the conserved motifs may change [Figure 4]. The novel virus species described in this thesis contain unique amino acids at positions that are conserved among all other members of the family *Bunyaviridae*. FERV and JONV have an asparagine residue at positions N<u>R</u>S$_{106}$ and N<u>R</u>A$_{130}$, respectively, whereas all other bunyaviruses have a Lysine (K) at the respective positions within the premotif A [Table IV.2]. The conserved amino acid Tryptophane (W) in motif A is replaced by Tyrosine in KIGV (K<u>Y</u>S$_{125}$) and by Phenylalanine in FERV (Q<u>F</u>S$_{139}$), respectively. Furthermore, in motif B KIGV S<u>T</u>S$_{208}$, JONV S<u>T</u>D$_{246}$ and FERV S<u>T</u>H$_{217}$ contain a Threonine while all bunyaviruses have a Serine (S) at the respective positions. These findings together with the experimental approaches on the insect specificity of FERV, GOLV, and JONV suggest that these amino acids might be characteristic for insect-specific bunyaviruses. The three herbeviruses HEBV, KIBV and TAIV shared all highly conserved RdRp protein motifs with bunyaviruses. Further functional studies are needed to investigate the effect of the amino acid changes in the highly conserved motifs of the insect specific bunyaviruses.

Table 3. Conserved motifs of the third conserved region of the RNA-dependent RNA polymerase protein of representative members of the family *Bunyaviridae*, ACC9.4 virus, Phasi-Charoen-like virus (PCLV) and bunyavirus isolates characterized in this thesis. Alignment was done using Expresso structural alignment available at T-Coffee web server (Tommaso et al., 2001). Abbreviations are *Hantaan virus* (HANV), *Puumala virus* (PUUV), *Thottapalayam virus* (TMPV), *Dugbe virus* (DUGV), *Crimean-Congo haemorrhagic fever virus* (CCHV), *Leopards hill virus* (LPHV), *Tomato spotted wilt virus* (TSWV), *Watermelon silver mottle virus* (WSMV), *Impatiens necrotic spot virus* (INSV), *Bunyamwera virus* (BUNV), *La Crosse virus* (LACV) = species *California encephalitis orthobunyavirus*, *Simbu virus* (SIMV), *Rift Valley fever virus* (RVFV), *Sandfly fever Naples virus* (SFNV), *Severe fever with Thrombocytopenia virus* (SFTSV), *Uukuniemi virus* (UUKV), Rice stripe virus (RSV), Kigluaik phantom virus (KIGV), European mountain ash ringspot-associated virus (EMARAV), Gouléako virus (GOLV), Herbert virus (HEBV), Kibale virus (KIBV), Taï virus (TAIV), Ferak virus (FERV), Jonchet virus (JONV).

Virus	Premotif A	Motif A	Motif B	Motif C	Motif D	Motif E
HANV*	IVRKYQRTEADRGFFITTLPTRCRLEIIEDYYDAIAKNISEEYISYGGEKKILAIQ	VSADATKWS	PD--GHHGEVKGNWLQGNLNKCSSLFGV	AHHSDDALFIY	IKISPKKTTVS	EFLSTF
PUUV	IVRKYQRTEADRGFFITTLPTRVRLEIIEDYYDAIAKVVPEEYISYGGERKILNIQ	VSADATKWS	PN--KVSASIKGNWLQGNLNKCSSLFGA	AHHSDDALFIY	IKISPKKTTVS	EFLSTF
TPMV	IVRKHQRTEADRGFFITTLPTRVRLEIIEDYFDSISKNVQEEYISYGGEKKILQIQ	VSADATKWS	SK--GRSGLVRGNWLQGNLNKCSSLFSV	AHHSDDALFIY	VKVSPKKTTVS	EFLSTF
DUGV*	LAPKAQLG-GSRDLLVQETGTKVIHATTEMFSRNLLKTTSDDGLTNPHL-KETR-L	ISGDNTKWG	ISKGIMAMNSYNHMGQGIHHATSSLLTS	AGSSDDYAKCI	MKDSAKTLVGD	EFYSEF
CCHV	LAPKAQLG-GARDLLVQETGTKVMHATTEMFSRNLLKTTSDDGLTNPHL-KETI-L	ISGDNTKWG	ISKGLMALNSYNHMGQGIHHATSSVLTS	AGSSDDYAKCI	MKDSAKTLVSD	EFYSEF
LPHV	LAPKAQLG-GHRDLLVQETGTKVIHAATEMFSRTLLSTTKDDGLTNNHL-KETI-L	ISGDNTKWG	VSKGKMALNSYNHMGQGIHHATSSVLTS	AGSSDDYAKCI	MKDSAKTLVSD	EFYSEF
TSWV*	VFEKMQRTKTDREIYLMSMKVKMMLYFIEHTFKHVAQSDPSEAISISGDNKIRALS	LSADQSKWS	LT--TNTYPVSMNWLQGNLNYLSSVYHS	IVHSDDNATSL	ITLNPKKSYAS	EFISER
WSMV	VFEKMQRTKMDREIYLMSMKTKMMLYFIEHTYKHIAQSDPSEAISISGDYKIKNLA	LSADQSKWS	LE--TNTFPVSMNWLQGNLNYLSSVYHS	MVHSDDNATSI	ITLNPKKSYAS	EFISER
INSV	VFEKMQRTKTDREIYLMSMKVKMMLYFIEHTFKHVAQSDPSEAISISGDNKIRALS	LSADQSKWS	LS--TNSYPVSMNWLQGNLNYLSSVYHS	IVHSDDNATSL	ITLNPKKSYAS	EFISER
BUNV*	FFNKGQKTAKDREIFVGEFEAKMCMYVVERISKERCKLNTDEMISEPGDSKLKILE	INADMSKWS	LN--YNYVQIKRNWLQGNFNYISSYVHS	MVHSDDNQTSL	CQANMKKTYIT	EFVSLF
LACV	FFNKGQKTSKDREIFVGEYEAKMCMYAVERIAKERCKLNPDEMISEPGDGKLKVLE	INADMSKWS	LN--SNTVLIKRNWLQGNFNYTSSYVHS	LVHSDDNQTSI	CQANMKKTYVT	EFVSLF
SIMV	FFNKGQKTAKDREIFVGEFEAKMCLYLVERISKERCKLNPEEMISEPGDGKLKKLE	INADMSKWS	LS--QNWVEIKRNWLQGNLNYTSSYLHS	MVHSDDNHTSI	NQANMKKTYIT	EFVSLF
RVFV*	LFKKQQHG-GLREIYVMGAEERIVQSVVETIARSIGKFFASDTLCNPPN-KVKIPE	TSDDARKWN	AFKGKTYLETTTGMMQGILHYTSSLLHT	MQGSDDSSMLI	IYPSEKSTANT	EYNSEF
SFNV	LFKKQQHG-GLREIYVMGADERIVQSIIEAIARAIGRFFDSDTLCNPTN-KTKIPE	TSDDARKWN	IDQGKTYLKTSTGMMQGILHFTSSLLHS	MQGSDDSSMII	IYPSEKSTPNT	EYNSEF
SFTSV	LFKKNQHG-GLREIYVMDANARLVQFGVETMARCVCELSPHETVANPRL-KNSIIE	SSNDAKKWN	MDKGRTYIKTETGMMQGILHFTSSLLHS	IEGSDDSAIMI	IYSSEKSTVNT	EYNSEF
UUKV	LFKKPQHG-GLREIYVLGFEERVVQLVIETIARQICKRFKSETLTNPKQ-KLAIPE	TSDDAAKWN	ISRGGAFVQTETGMMQGILHYTSSLLHT	LQSSDDSGMMI	IYSSVKSTNNT	EFNSEF
PCLV	IFRKPQHG-GDREIYVLGFQERVVQRLIEQISRIICRFIPEETMTHPGN-KIAIPE	SSADASKWS	IRAGSPYIEVTTGMMQGILHYTSSLYHS	LQSSDDSYFSV	VVNSIKTVLNS	EFNSNF
ACC9.4	LFKKNQHG-GLREIYVLTIKSKLLALFLETCSRCLCEQFTVETMTHPDC-KMEVIE	CSADKKSWN	PNAGSGYCILHRGMMQGILHYTSSLLHV	MCSSDDSATIM	FTNSEKSVMGS	EFNSEF
RSV*	IFKKNQHG-GLREIYVLNIFERIMQKTVEDFSRAILECCPSETMTSPKN-KFRIPE	TSDDASKWN	MKAGRSYIETETGMMQGILHYTSSLFHA	MESSDDSSFII	IYKSPKSTTQT	EFNSEF
KIGV**	MSAKDQRG-SGRPIATPDLGTKAALMMIEKPEAAKGAFVGNNITVAGKE-KLR---	LTEDQSKYS	RDEKSLGIRAIIGWPQGMLNDISTSVHS	LVHSDDSWVVV	LKLNEKKLWGS	ELVSNY
EMARAV*	IFNKDQRTTDDREIYTGNAQVRLCLYPLEMTFKSICKKIPEEAITISGDQKQRKLL	VSSDASKWS	FT--QNWFNVRSNWLQGNLNMTSSFVHH	MVHSDDSTYDF	ITLNEKKTYIS	EFLSTT
GOLV**	**IFKKNQHG-GLREIYVLDLASRIVQLCLEEISRAVCQELPIEMMHPEL-KLKKPQ**	**SSNDAKVWN**	**LRPGETFMRIESGMMQGILHYTSSLFHA**	**LVSSDDSSRMT**	**IWMSPKSTYCC**	**EFNSEY**
HEBV**	**LFCKDQRTAKDREIYEMELEGKILLYVIERLFKTYSREDMNEMISRPGDVKVLDIE**	**INADMSKWS**	**LS--TNCVTISQNWFQGNLNYMSSFCHS**	**LVHSDDNQTGV**	**FILNTKKTYIS**	**EFISMH**
KIBV	**LFCKDQRTAKDREIYEMELEGKILLYVIERLFKTYSREDMNEMISRPGDVKVLDIE**	**INADMSKWS**	**LS--SNCVKITQNWFQGNLNYMSSFCHS**	**LVHSDDNQTGV**	**FILNTKKTYIS**	**EFISMH**
TAIV	**LFCKDQRTAKDREIYEMELEGKILLYVIERLFKTYSREDMNEMISRPGDVKVLDIE**	**INADMSKWS**	**LS--TNCVKISQNWFQGNLNYLSSFCHS**	**LVHSDDNQTGV**	**FILNTKKSFIS**	**EFISMH**
FERV**	**MSEKEQRG-GGRPIGSADFFTKQRLYCIEMIYQRIGQAQGENLMAKKVN-RSAKLS**	**IVMDQSQFS**	**LTKD-GGIRGVAGWVQGMLNISSTHIHI**	**LVNSDDSFAVI**	**LKQNKKKSYMS**	**EVIQKY**
JONV**	**TSSKQQRG-KGRQIASADFYTKNGLHCIEEAYKSQSAKDETNLLRSGVN-RARAVS**	**LVEDQTKWS**	**ITEN-GAILGRIGWMQGMLNFTSTDCAK**	**SLNSDDSFHAV**	**MKLNIKKSYIS**	**EMIQLY**

3'vRNA binding site

Nucleotide addition site

*prototype species **proposed prototype species

Infection experiments *in vitro* are useful tools to evaluate the host range of novel virus isolates. Cell culture infection experiments with insect-specific or vertebrate-pathogenic mosquito and tick-borne flaviviruses of the "no vector group" were in agreement with the natural host range and phylogenetic clustering of the viruses (Kuno, 2007). For FERV, GOLV, HEBV and JONV the infection of a broad variety of vertebrate cells, like reptile, bird and mammalian cells, showed no virus replication after several passages onto fresh cells. Moreover it has been shown that temperature sensitivity is a factor limiting the host range of a virus (Aliota & Kramer, 2012). Temperature sensitivity experiments on susceptible insect cells with FERV, GOLV, HEBV and JONV showed complete abrogation of viral growth at vertebrate typical temperatures. The incapability of replication at the body temperature of vertebrates (approx. 36.5-42 °C) significantly reduces the likelihood of the respective viruses to be able to infect vertebrates. These findings provide futher support for the *in vitro* vertebrate infection experiments. In conclusion, gouko-, herbe-, jon-, and feraviruses are likely to represent four distinct lineages of insect-restricted viruses.

Chung and coworkers detected GOLV and HEBV sequences in South Korean pigs (*Sus scrofa domestica*) (Chung *et al.*, 2014). Both viruses were claimed to cause prevalent and highly lethal infections in farmed pigs. These conclusions were highly controversial with our findings on host limitations of GOLV and HEBV and were therefore addressed in this thesis. Serum samples collected from *Sus scrofa domestica* found in the village of Gouléako in Ivory Coast where GOLV and HEBV were detected for the first time were investigated. The sample set was expanded to samples from mosquito-exposed pigs from Ghana, a neighbouring country to Ivory Coast where HEBV was also detected in mosquitoes (Marklewitz *et al.*, 2013). All samples were investigated for the presence of viral nucleic acids and antibodies against GOLV and HEBV. No viral genome copies of GOLV and HEBV, as well as antibodies were found in pigs (Junglen *et al.*, 2015). Comparison of the short viral glycoprotein sequence fragment from Korean pigs with the 26 GOLV and 36 HEBV strains isolated from mosquitoes revealed that the Korean sequences fall within the species diversity of GOLV or HEBV and do not represent phylogenetic outliers. These results do not suggest that the Korean strains mutated and became pathogenic to vertebrates. However, functional domains that might facilitate changes in host tropism could not be investigated on the data provided by Chung and coworkers as the respective regions of the glycoproteins were not sequenced. In theory, the acquisition of a glycoprotein from a closely related virus with a vertebrate tropism could potentially provide the genetic basis for an infection of pigs. The closest related virus showing a vertebrate tropism is SFTSV and reassortment is believed to occur only within a virus of the same species. Thus, it is very unlikely that GOLV and HEBV are responsible for the death of Korean farmed pigs.

Only for the genus *Flavivirus* within the family *Flaviviridae*, an evolution from insect-specific to dual host viruses has been suggested as the insect-specific flaviviruses branch deeper in the phylogenetic tree than the arthropod-borne flaviviruses (Cook *et al.*, 2012). *Herbe-*, *Gouko-*, *Fera-* and *Jonviruses* define four distinct deep branching lineages that share

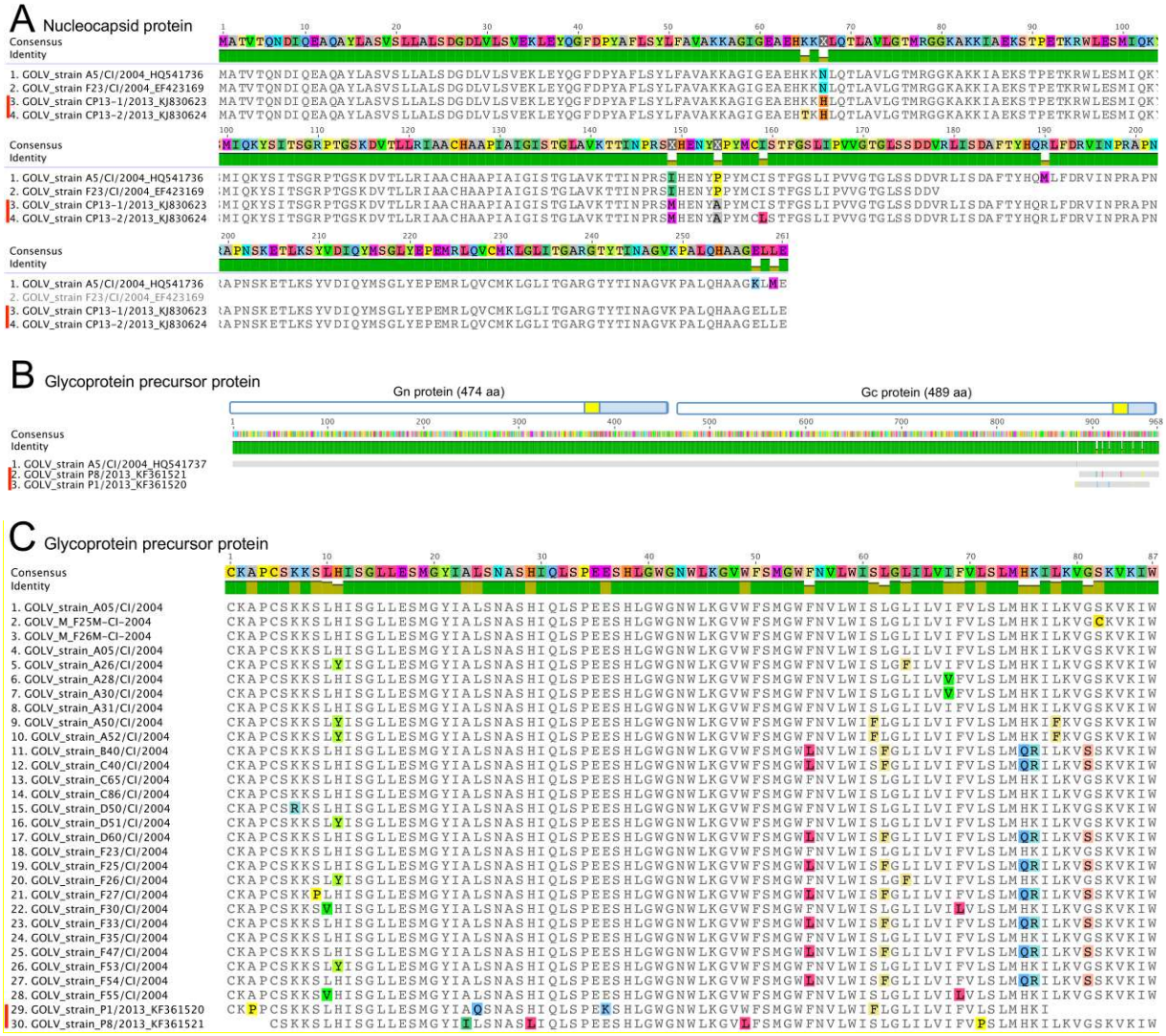
common ancestors with all bunyaviruses except nairoviruses. The experimentally validated insect-restriction of FERV, GOLV, HEBV and JONV was used in ancestral trait reconstruction analysis and maximum likelihood hypothesis testing to investigate the evolution of host tropism in the family *Bunyaviridae*. Due to the fact that the vertebrate host hypothesis was rejected in all deep sister nodes, the vertebrate or dual host tropism must have evolved several times convergently during bunyavirus evolution. As another outcome of this analysis the exclusively vertebrate infecting genus *Hantavirus* seems to have lost the ability to infect arthropods over time, rather than preserving vertebrate mono-tropism from ancestral viruses. This development may have happened via a dual host intermediate stage where the potential transmission of HANV through mites could be subject to speculation (Yu & Tesh, 2014).

The rapid development of deep sequencing technologies and analysis tools allows the generation and analysis of entire transcriptome data sets for presence of viral sequences. A great diversity of novel bunyaviruses in arthropods was discovered recently (Li *et al.*, 2015; Tokarz *et al.*, 2014). In total, 112 *bona fide* novel viruses were discovered in 70 arthropod species (Li *et al.*, 2015). Bunyaviruses were for the first time detected in non-blood-feeding arthropods confirming the hypothesis of an arthropod origin of the family. Further members of the putative new bunyavirus genera *Gouko-*, *Herbe-*, *Fera-* and *Jonvirus,* as well as of the proposed genus *Phasmavirus* and the unassigned genera *Emara-* and *Tenuivirus* have been discovered by other groups (Reviewed in Junglen, 2016).

This thesis is based on live virus isolates which enabled a comprehensive phenotypic and molecular characterization of four putative new bunyavirus genera. The generated data have shed new light on the genetic diversity, the origin and the evolution of the largest RNA virus family. The proposed new genera almost double the existing number of bunyavirus genera. The viruses were isolated from mosquitoes originating from a single tropical rainforest region suggesting that more diverse bunyaviruses exist in other geographic regions and arthropod species. The knowledge about the genome organization of insect-specifc bunyaviruses might help to understand the emergence of vertebrate-pathogenic bunyaviruses.

6. SUPPLEMENTARY INFORMATION

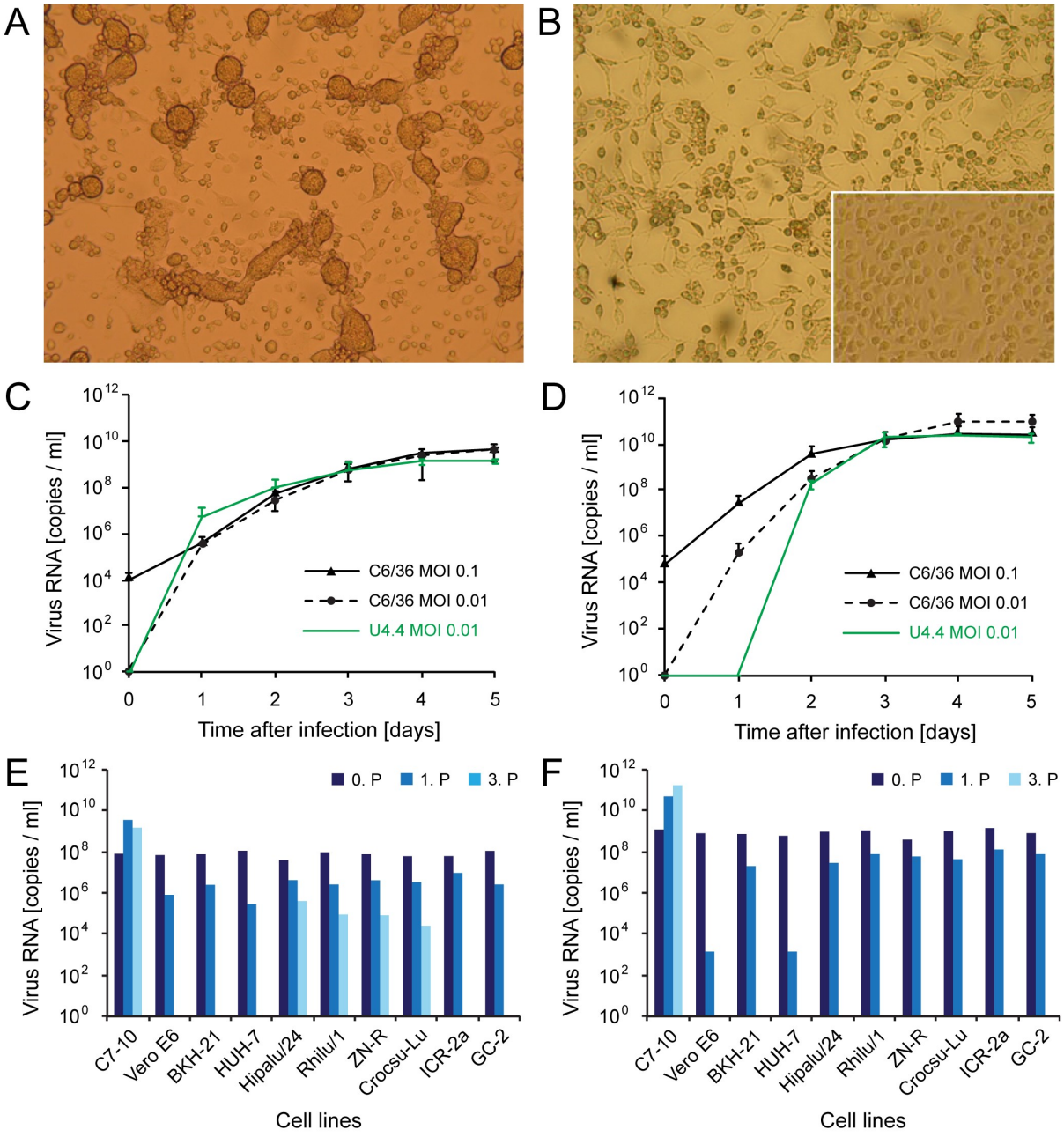
Supplementary material of Chapter III)



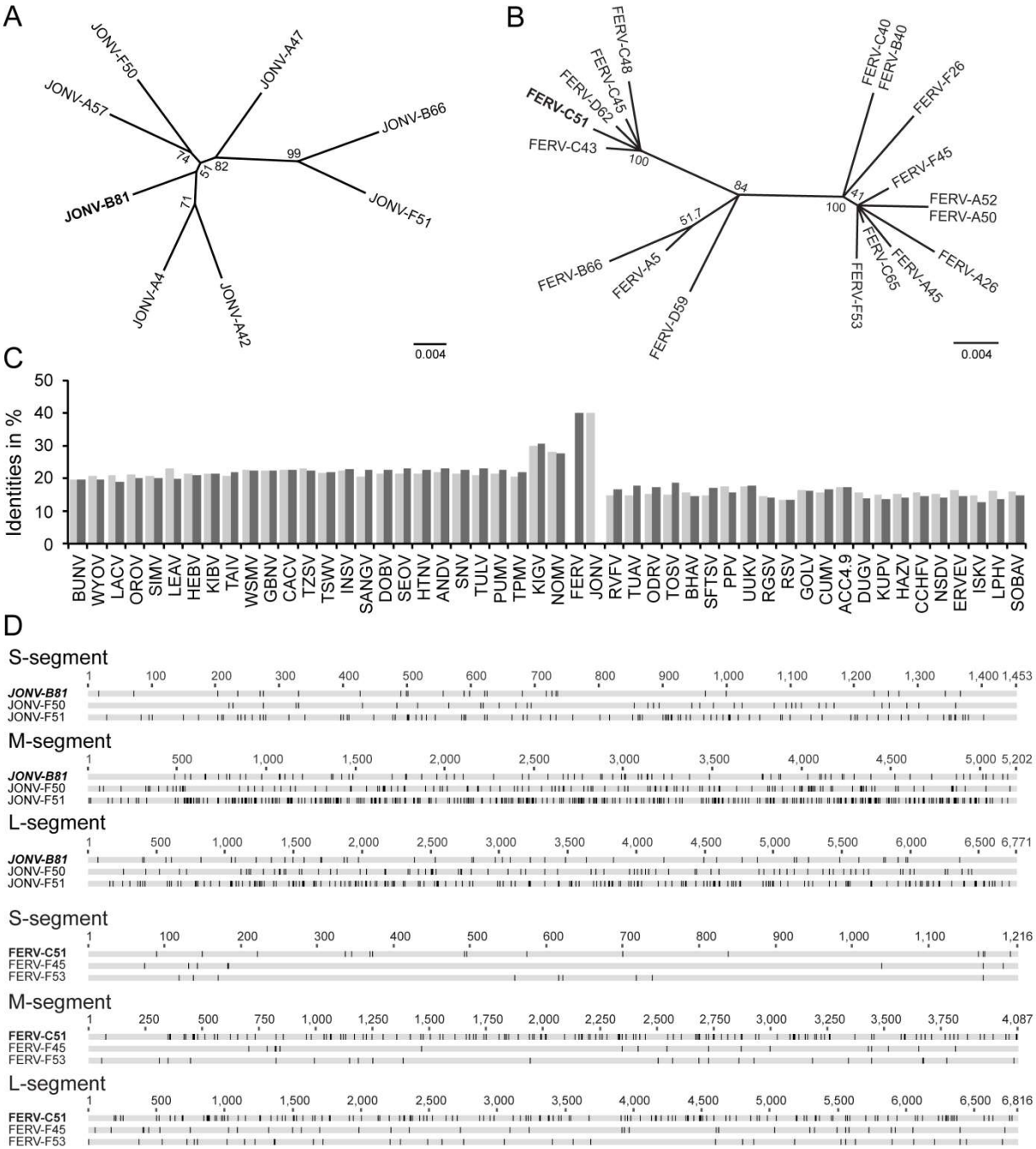
Supplement Figure III.1 Alignment of Gouléako virus (GOLV) strains from mosquitoes originating from Côte d'Ivoire and Ghana and swine sampled in Korea. A) Alignment of the GOLV nucleocapsid proteins; B) alignment of complete GOLV glycoprotein precursor protein from mosquito and of protein fragments identified in swine. Schematic overview of encoded proteins is shown in boxes. Transmembrane domains are marked in yellow. Protein domains located outside virions are shown in dark blue, and those located inside virions are in light blue. C) Alignment of Gc proteins originating from mosquitoes and swine. A red line (at consensus identity 29 and 30) marks sequences from swine published by Chung et al. in 2014 (Chung *et al.*, 2014).

Supplement Figure III.2 (Continued)

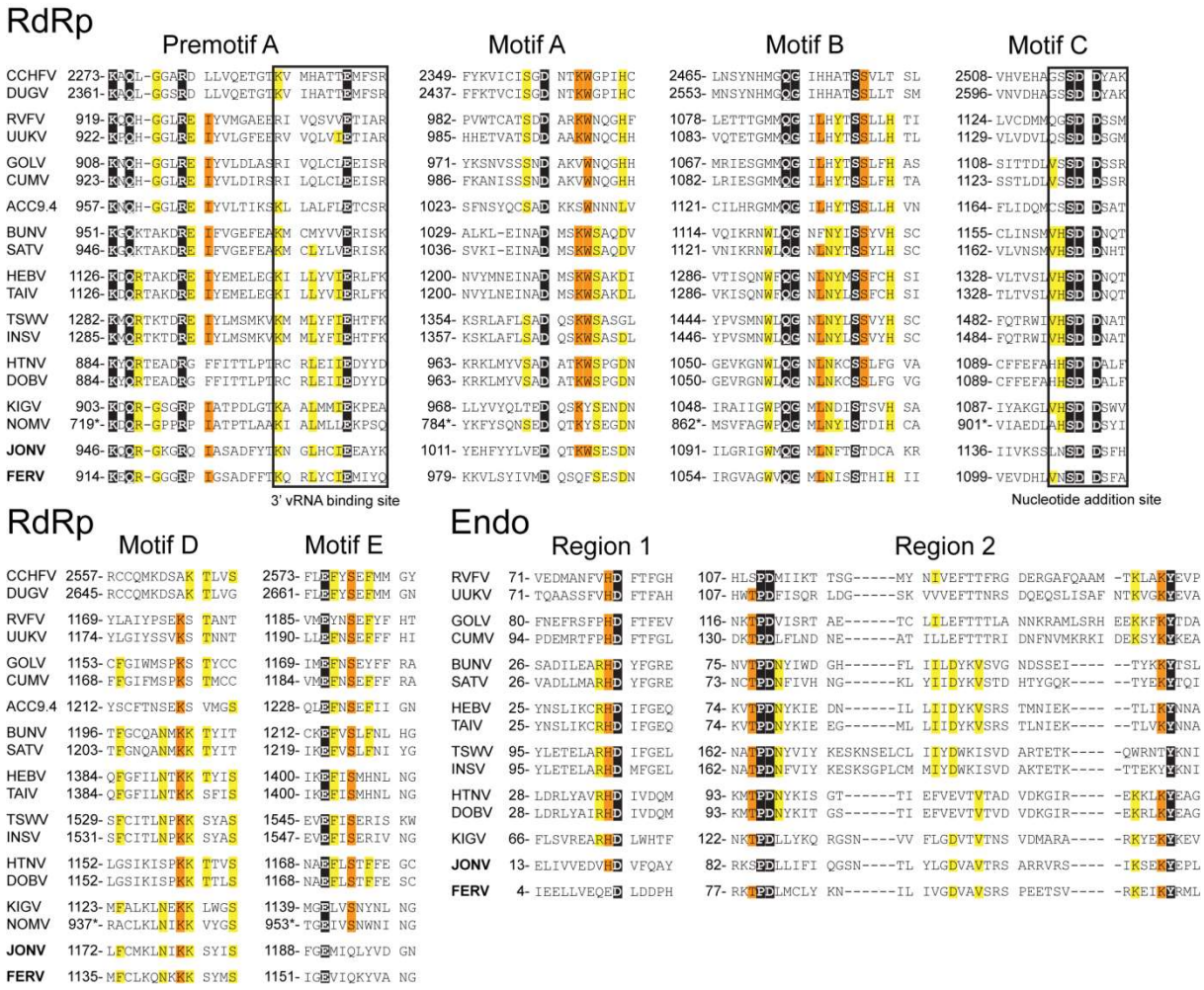
Supplement Figure III.2 Alignment of Herbert virus (HEBV) strains from mosquitoes and pigs. A) Overview of location of amplified RNA-dependent RNA polymerase (RdRp) protein sequences available from mosquitoes and swine. Sequences detected in swine in South Korea are indicated by a red line. B) Alignment of RdRp protein sequences from mosquitoes and swine. Amino acid changes are colored. Sequences detected in swine in South Korea are indicated by a red line. C) Alignment of HEBV protein sequences of the third conserved region of the RdRp identified in mosquitoes from Côte d'Ivoire and Ghana.



Supplement Figure IV.1 Growth and cytopathic effects of JONV and FERV. (A and B) Cytophatic effects of JONV-infected (A) and FERV-infected (B) C6/36 insect cells 5 dpi. Mock-infected cells are shown in a smaller picture in B. (C and D) Growth analysis of JONV (C) and FERV (D) on C6/36 cells infected at MOIs of 0.1 and 0.01. U4.4 cells were infected at an MOI of 0.01. The genome copy numbers per milliliter cell culture supernatants were measured for 5 d by real-time RT-PCR. (E and F) Growth of JONV (E) and FERV (F) on insect (C7/10) and different vertebrate cells. Cells were infected at an MOI of 1, and three blind passaged were performed after 7 dpi. Genome copy numbers in cell culture supernatants were measured via real-time RT-PCR after passages 0, 1, and 3.



Supplement Figure IV.2 Phylogenetic relationship and single-nucleotide polymorphisms of JONV and FERV isolates, as well as genetic distances of JONV and FERV to other bunyaviruses. (A and B) Maximum likelihood analyses of JONV (A) and FERV (B) isolates performed on a MAFFT-E nucleotide alignment comprising the conserved palm domains of the RdRp gene. (C) Genetic distances of JONV and FERV to representative bunyaviruses based on a structural alignment of RdRp proteins. (D) Full-genome analysis of single-nucleotide polymorphisms of JONV and FERV isolates. Nucleotide mutations are illustrated as black bars. Nucleotide substitutions (nonsynonymous and synonymous) were determined for the N and NSs protein ORFs, using the Datamonkey webserver (Delport *et al.*, 2010). Mean dN-dS ratio estimation was performed using the SLAC codon-based maximum likelihood method and HKY85 substitution model. NSs and N protein ORFs had similar values (JONV NSs = −0,919; n = −0,919; FERV NSs = −1; n = −1; FERV NSs = −1).

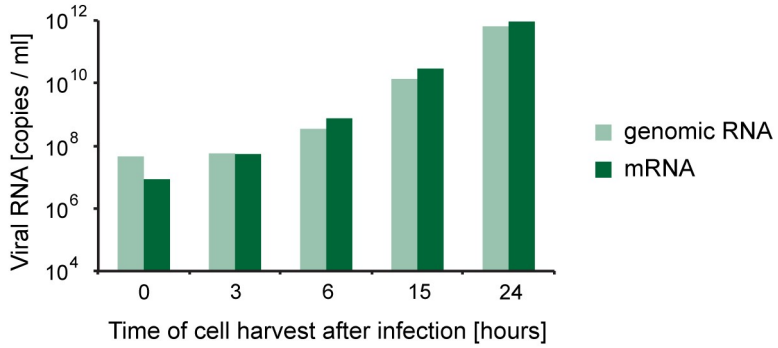


Supplement Figure IV.3 Multiple-sequence alignments of conserved motifs of the RNA-dependent RNA polymerase (RdRp) and the endonuclease (Endo). Alignments of JONV and FERV with representative bunyaviruses were performed using Expresso in T-Coffee (Notredame *et al.*, 2000). Amino acids with 100% similarity are highlighted in black, those with 75% similarity in orange, and those with 50% similarity in light yellow. Conserved functional domains are annotated. Numbers represent genome positions.

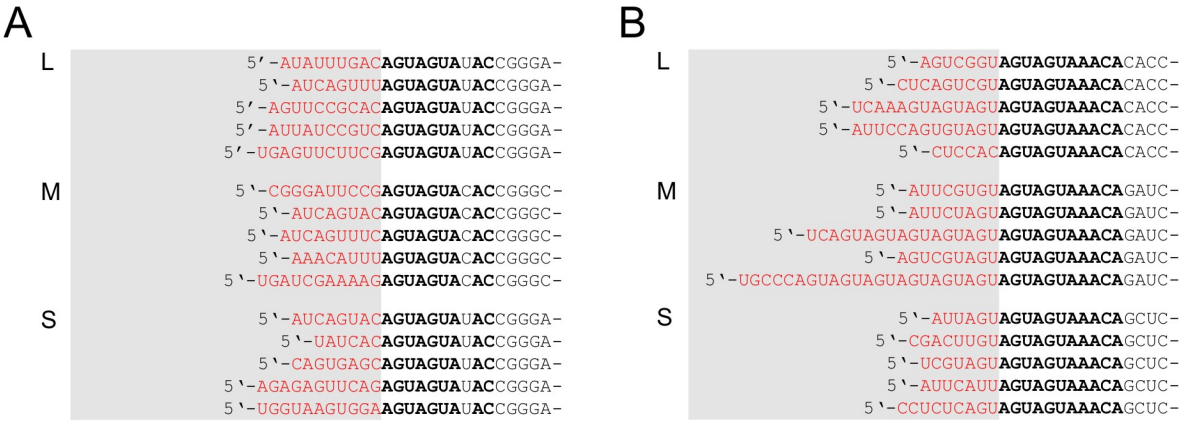
Supplement Figure IV.4 (Continued)

Supplement Figure IV.4 (Legend continued on following page)

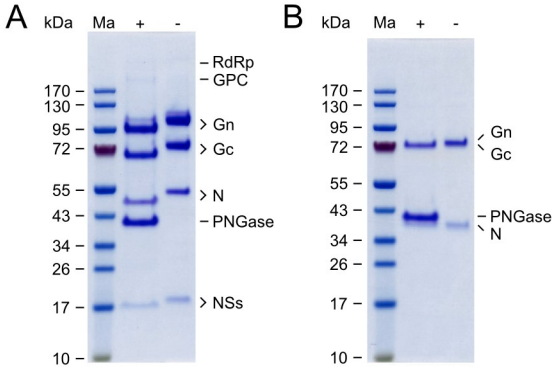
Alignment of polymerase protein sequences of bunyaviruses, arenaviruses, and orthomyxoviruses. Bunyavirusand arenavirus L segment sequences were aligned with concatenated orthomyxo PA and pb1 segment sequences using the Expresso structural alignment algorithm on the TCoffee webserver (Notredame *et al.*, 2000). The alignment was reduced manually to conserved columns. Black, 100% similar; orange, 80–100% similar; yellow, 60–80% similar; white, less than 60% similar.



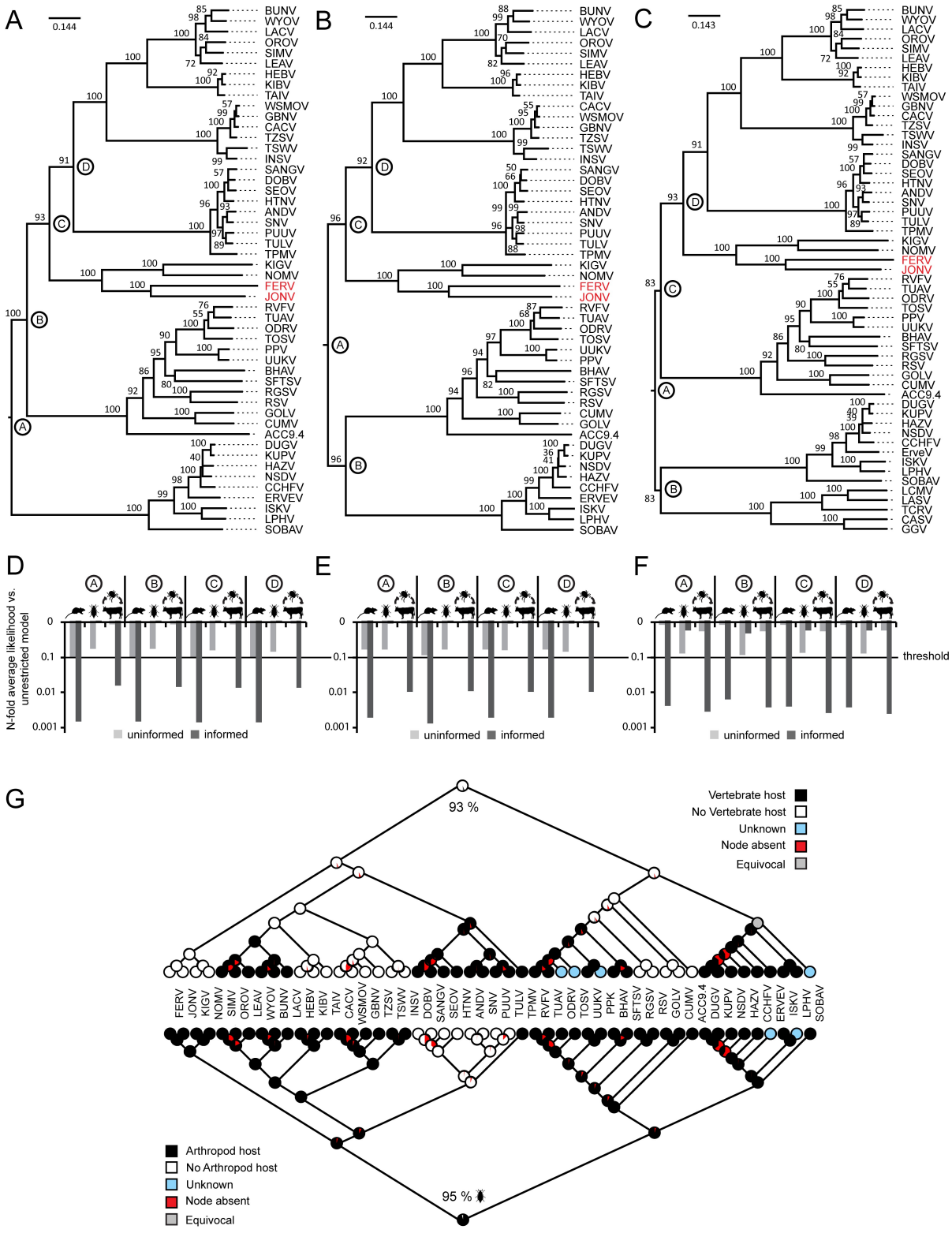
Supplement Figure IV.5 RNA species during JONV replication in insect cells. Total RNA was extracted at 0, 3, 6, 15, and 24 hpi from C6/36 cells infected with JONV at a MOI of 1. Strain-specific cDNA was synthesized, and amounts of positive and negative sense viral RNA were quantified by real-time RT-PCR.



Supplement Figure IV.6 Nontemplated sequences of JONV and FERV mRNAs. 5' genome termini of L, M, and S segment mRNAs of JONV (A) and FERV (B) are shown. Total RNA was extracted at 1 dpi from FERV- and JONV-infected C6/36 cells, respectively. Genome termini were amplified using 5' RACE-PCR, PCR products were cloned, and five randomly chosen clones were analyzed. Putative transcription primers obtained from host cell mRNAs (nontemplated sequences) are marked in red. Conserved genome termini of FERV and JONV are shown in bold.



Supplement Figure IV.7 N-linked glycosylation analysis of JONV and FERV proteins. Analysis of JONV (A) and FERV (B) proteins either treated with (+) Peptide-N-glycosidase F or not treated (–). Proteins were separated by SDS/PAGE and stained with Coomassie blue.



Supplement Figure IV.8 Phylogenetic reconstructions based on alternative bunyavirus tree topologies. (A–C) Maximum likelihood phylogeny of conserved L protein domains of bunyaviruses with nairoviruses as a defined basal taxon (A) or midpoint rooted with phleboviruses and nairoviruses as sisterclades (B), or midpoint rooted with arenaviruses and nairoviruses as basal sister taxa (C). Bootstrap values of 1,000 replicates are indicated at tree nodes. (D–F) Probalistic host reconstructions for the tree topologies shown in A–C. Hypothesized (fossilized) ancestral host assumptions at deep tree nodes A–D are symbolized by vertebrate and arthropod silhouettes. Bars represent the resulting loss of likelihood of trait change models conferred by fossilization (averaged results over 1,000 bootstrap tree replicates). The significance threshold was 10-fold loss of likelihood. (G and H) Parsimony-based ancestral

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reconstructions of arthropod and vertebrate host associations for tree topology shown in A and B. For parsimony-based ancestral reconstructions based on tree topology shown in C, refer to Fig. 5 A and B. LCMV is the type species of the family *Arenaviridae*. Arenaviruses chronically infect rodents and snakes and are transmitted by contact with excretions. Abbreviations (and GenBank accession numbers) are as follows: ACC9.4, uncultured virus isolate acc_9.4 (KF298274); ANDV, Andes virus (NC_003468); BHAV, Bhanja virus (JX961619); BUNV, Bunyamwera virus (NC_001925); CACV, capsicum chlorosis virus (NC_008302); CASV, CAS virus (NC_018484); CCHFV, Crimean-Congo hemorrhagic fever virus (NC_005301); CUMV, Cumuto virus (KF543244); DOBV, Dobrava virus (NC_005235); DUGV, Dugbe virus (NC_004159); ERVEV, Erve virus (JF911697); FLUAV, influenza A virus (NC_002021); FLUBV, influenza B virus (NC_002204); FLUCV, influenza C virus (NC_006308); GBNV, groundnut bud necrosis virus (NC_003614); GGV, Golden Gate virus (NC_018482); GOLV, Gouléako virus (HQ541738); HAZV, Hazara virus (DQ076419); HEBV, Herbert virus (JQ659256); HTNV, Hantaan virus (NC_005222); INSV, impatiens necrotic spot virus (NC_003625); ISKV, Issyk-Kul virus (KF892055); KIBV, Kibale virus (KF590577); KIGV, Kigluaik phantom virus (KJ434182); KUPV, Kupe virus (EU816899); LACV, La Crosse virus (NC_004108); LASV, Lassa virus (NC_004297); LCMV, Lymphocytic choriomeningitis virus (NC_004291); LEAV, Leanyer virus (HM627178); LPHV, Leopards Hill virus (AB842091); NOMV, Nome phantom virus (KJ434185); NSDV, Nairobi sheep disease virus (EU697949); ODRV, Odrenisrou virus (HM566174); OROV, Oropouche virus (NC_005776); PPV, precarious point virus (HM566181); PUUV, Puumala virus (NC_005225); RGSV, rice grassy stunt virus (NC_002323); RSV, rice stripe virus (NC_003755); RVFV, Rift Valley fever virus (NC_014397); SANGV, Sangassou virus (JQ082302); SEOV, Seoul virus (NC_005238); SFTSV, severe fever with thrombocytopenia syndrome virus (NC_018136); SIMV, Simbu virus (NC_018476); SNV, Sin Nombre virus (NC_005217); SOBAV, South Bay virus (KM048320); TAIV, Taï virus (KF590574); TCRV, Tacaribe virus (NC_004292); THOV, Thogoto virus (NC_006495); TPMV, Thottapalayam virus (NC_010707); TOSV, Toscana virus (NC_006319); TSWV, tomato spotted wilt virus (NC_002052); TULV, Tula virus (NC_005226); TUAV, Turuna virus (HM119431); TZSV, tomato zonate spot virus (NC_010491); UUKV, Uukuniemi virus (NC_005214); WSMOV, watermelon silver mottle virus (NC_003832); WYOV, Wyeomyia virus (JN572080).

Supplement Table IV.1 Trait matrix used for ancestral reconstructions.

Virus	Uninformed		Informed	
	Arthropod	Vertebrate	Arthropod	Vertebrate
BUNV	1	1	1	1
WYOV	1	1	1	1
LACV	1	1	1	1
OROV	1	1	1	1
SIMV	1	1	1	1
LEAV	1	1	1	1
HEBV	1	n.k.	1	0
KIBV	1	n.k.	1	0
TAIV	1	n.k.	1	0
WSMOV	1	0	1	0
GBNV	1	0	1	0
CACV	1	0	1	0
TZSV	1	0	1	0
TSWV	1	0	1	0
INSV	1	0	1	0
SANGV	0	1	0	1
DOBV	0	1	0	1
SEOV	0	1	0	1
HTNV	0	1	0	1
ANDV	0	1	0	1
SNV	0	1	0	1
PUUV	0	1	0	1
TULV	0	1	0	1
TPMV	0	1	0	1
KIGV	1	n.k.	1	0
NOMV	1	n.k.	1	0
FERV	1	n.k.	1	0
JONV	1	n.k.	1	0

RVFV	1	1	1	1
TUAV	1	n.k.	1	n.k.
ODRV	1	n.k.	1	n.k.
TOSV	1	1	1	1
PPV	1	n.k.	1	n.k.
UUKV	1	1	1	1
BHAV	1	1	1	1
SFTSV	1	1	1	1
RGSV	1	0	1	0
RSV	1	0	1	0
GOLV	1	n.k.	1	0
CUMV	1	n.k.	1	0
ACC9.4	1	n.k.	1	0
DUGV	1	1	1	1
KUPV	1	1	1	1
HAZV	1	1	1	1
NSDV	1	1	1	1
CCHFV	1	1	1	1
ERVEV	n.k.	1	n.k.	1
ISKV	1	1	1	1
LPHV	n.k.	1	n.k.	1
SOBAV	1	n.k.	1	n.k.
LCMV	0	1	0	1
LASV	0	1	0	1
TCRV	1	1	1	1
CASV	n.k.	1	n.k.	1
GGV	n.k.	1	n.k.	1

n.k. = not known. Abbreviations (and GenBank accession numbers) are as follows: ACC9.4, uncultured virus isolate acc_9.4 (KF298274); ANDV, Andes virus (NC_003468); BHAV, Bhanja virus (JX961619); BUNV, Bunyamwera virus (NC_001925); CACV, Capsicum chlorosis virus (NC_008302); CCHFV, Crimean-Congo hemorrhagic fever virus (NC_005301); CUMV, Cumuto virus (KF543244); DOBV, Dobrava virus (NC_005235); DUGV, Dugbe virus (NC_004159); ERVEV, Erve virus (JF911697); GBNV, groundnut bud necrosis virus (NC_003614); GOLV, Gouléako virus (HQ541738); HAZV, Hazara virus (DQ076419); HEBV, Herbert virus (JQ659256); HTNV, Hantaan virus (NC_005222); INSV, Impatiens necrotic spot virus (NC_003625); ISKV, Issyk-Kul virus (KF892055); KIBV, Kibale virus (KF590577); KIGV, Kigluaik phantom virus (KJ434182); KUPV, Kupe virus (EU816899); LACV, La Crosse virus (NC_004108); LEAV, Leanyer virus (HM627178); LPHV, Leopards Hill virus (AB842091); NOMV, Nome phantom virus (KJ434185); NSDV, Nairobi sheep disease virus (EU697949); ODRV, Odrenisrou virus (HM566174); OROV, Oropouche virus (NC_005776); PPV, Precarious point virus (HM566181); PUUV, Puumala virus (NC_005225); RGSV, rice grassy stunt virus (NC_002323); RSV, rice stripe virus (NC_003755); RVFV, Rift Valley fever virus (NC_014397); SANGV, Sangassou virus (JQ082302); SEOV, Seoul virus (NC_005238); SFTSV, severe fever with thrombocytopenia syndrome virus (NC_018136); SIMV, Simbu virus (NC_018476); SNV, Sin Nombre virus (NC_005217); SOBAV, South Bay virus (KM048320); TAIV, Taï virus (KF590574); TPMV, Thottapalayam virus (NC_010707); TOSV, Toscana virus (NC_006319); TSWV, tomato spotted wilt virus (NC_002052); TULV, Tula virus (NC_005226); TUAV, Turuna virus (HM119431); TZSV, tomato zonate spot virus (NC_010491); UUKV, Uukuniemi virus (NC_005214); WSMOV, watermelon silver mottle virus (NC_003832); WYOV, Wyeomyia virus (JN572080).

7. ABBREVIATIONS

#	°C	degree Celsius
	3'	three prime end
	5'	five prime end
A	aa	amino acid
	Ae	*Aedes aegypti*
	AG	Aktiengesellschaft
	agRNA	antigenomic RNA
	AGUV	Aguacate virus
	AINOV	Aino virus
	AKAV	Akabane virus
	AMBV	Anhembi virus
	ANDV	Andes virus
B	BATV	Batai virus
	BeNMV	bean necrotic mosaic virus
	BHK	baby hamster kidney
	BHK-21	baby hamster kidney *(cell line)*
	BRZV	Brazoran virus
	BUNV	Bunyamwera virus
C	C6/36	*Aedes albopictus (cell line)*
	C7/10	*Aedes albopictus (cell line)*
	CACV	Capsicum chlorosis virus
	CCHFV	Crimean-Congo hemorrhagic fever virus
	cDNA	complementary DNA
	CDUV	Candiru virus
	CI	Côte d'Ivoire
	CPE	cytopathic effect
	cRNA	coding RNA
	Crocsu-Lu	Lesser white-toothed shrew lung *(cell line)*
	CUMV	Cumuto virus
	CYV	Culex Y virus
D	d	days
	DAPI	4',6-diamidino-2-phenylindole
	DI	defective interfering
	DIG	digoxigenin
	DNA	deoxyribonucleic acid
	DOBV	Dobrava virus
	dpi	days postinfection
	dsDNA	double-stranded DNA
	dsRNA	double-stranded RNA
E	EM	electron microscopy
	EMARAV	European mountain ash ringspot-associated virus

	Endo	endonuclease
	ER	endoplasmatic reticulum
	EVE	endogeneous viral elements
F	FAM	carboxyfluorescein
	FERV	Ferak virus
	FITC	fluorescein isothiocyanate
G	g	grams
	GBNV	groundnut bud necrosis virus
	Gc	carboxy terminal glycoprotein
	Gn	aminoterminal glycoprotein
	GOLV	Gouléako virus
	GPC	glycoprotein precursor
	gRNA	genomic RNA
	GRSV-TCSV	groundnut ringspot and tomato chlorotic spot virus reassortant
H	h	hours
	HEBV	Herbert virus
	HEK	human embryonic kidney
	HipaLu/24	Aba roundleaf bat lung cells *(cell line)*
	hpi	hours postinfection
	HTNV	Hantaan virus
	HuH	human hepatocellular carcinoma
	HuH-7	human hepatocellular carcinoma *(cell line)*
	HVZ10	Hantavirus Z10 virus
I	IAV	intracellular annular viruses
	ICR-2a	grass frog embryos *(cell line)*
	ICTV	International Committee on Taxonomy of Viruses
	IDV	intracellular dense viruses
	IFA	immunofluorescence assay
	IFN	type I interferon
	IIFA	indirect immunofluorescence assay
	INSV	Impatiens necrotic spot virus
	ISG15	interferon stimulated gene product 15
J	JONV	Jonchet virus
K	kDa	kilodalton *(molecular mass)*
	KIBV	Kibale virus
	KIGV	Kigluaik phantom virus
	KNUST	Kwame Nkrumah University of Science and Technology
L	L	large
	L929	mouse fibroblast *(cell line)*
	LACV	La Crosse virus
	LC-MS	liquid chromatography mass spectrometry
	LEAV	Leanyer virus

M	M	medium
	MALDI-TOF	matrix-assisted laser desorption ionization-time of flight
	MCAV	Macaua virus
	Mda5	melanoma differentiation antigen 5
	MEF	mouse embryo fibroblast *(cell line)*
	ML	Maximum-likelihood
	ml	milliliter
	MOI	multiplicity of infection
	MRCA	most recent common ancestor
	mRNA	messenger RNA
	MYSV	melon yellow spot virus
N	N	nucleocapsid
	NCR	noncoding regions
	NJ	neighbor-joining
	nm	nanometre
	NOMV	Nome phantom virus
	NRIV	Ngari virus
	NS	nonstructural
	NSm	nonstructural M
	NSs	nonstructural S
	nt	nucleotide
O	ORF	open reading frame
	OROV	Oropouche virus
	OTU	ovarian tumor
P	PBS	phosphate-buffered saline
	PCLV	Phasi-Charoen-like virus
	PK	porcine kidney
	PSEK	porcine stable equine kidney *(cell line)*
	PTV	Punta Toro virus
	PUUV	Puumala virus
R	RACE-PCR	rapid amplification of cDNA ends-PCR
	RdRp	RNA-dependent RNA polymerase
	RGSV	Rice grassy stunt virus
	RHBV	Rice hoja blanca virus
	RhiLu/1	horseshoe bat lung cells *(cell line)*
	RIG-I	retinoic acid-inducible gene
	RNA	ribonucleic acid
	RNA1	*'smallest segment of tenuiviruses'*
	RNA2	*'second largest segment of tenuiviruses'*
	RNA3	*'third largest segment of tenuiviruses'*
	RNAi	RNA interference
	RNP	ribonucleoprotein
	RSV	Rice stripe virus
	RT-PCR	reverse transcription-PCR
	RVFV	Rift Valley fever virus

S	S	small
	SATV	Sathuperi virus
	SBV	Schmallenberg virus
	SEOV	Seoul virus
	SFCV	Sandfly fever Cyprus virus
	SFG	Sandfly fever group
	SFNV	Sandfly fever Naples virus
	SFTSV	Severe fever with thrombocytopenia virus
	sg mRNA	subgenomic mRNA
	SHAV	Shamonda virus
	SIMV	Simbu virus
	siRNA	small interfering RNA
	SNV	Sin Nombre virus
	SORV	Sororoca virus
	SP	signal peptides
	sp.	species
	spp.	species (*Plural*)
	ssRNA	single-stranded RNA
T	TAIV	Taï virus
	TBK1	TANK-binding kinase 1
	$TCID_{50}$	50% tissue culture infective dose
	TMD	transmembrane domain
	TOSV	Toscana virus
	TPMV	Thottapalayam virus
	TSWV	Tomato spotted wilt virus
	TULV	Tula virus
	TZSV	Tomato zonate spot virus
U	UTR	untranslated region
	UUKV	Uukuniemi virus
V	VeroE6/7	*Cercopithecus aethiops* kidney *(cell line)*
	vol	volumen
	VP3	viral protein 3
	vRNA	virion-sense RNA
	VSV	Vesicular stomatitis virus
W	WMV	Wheat mosaic virus
	WNV	West Nile virus
	WSMOV	Watermelon silver mottle virus
	WYOV	Wyeomyia virus
	ZN-R	goat kidney *(cell line)*

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